UNIVERSITY OF TASMANIA

Application of microsatellite markers to the genetic improvement of *Acacia* in Vietnam

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Declaration

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Abstract

Acacia plantations and downstream wood processing industries make a major contribution to the Vietnamese economy. In 2014, Vietnam exported an estimated 5.4M ton of wood chip valued at about $300M and most of this from acacia plantations. These are mainly *A. mangium* from open-pollinated seed and cloned natural hybrid with *A. auriculiformis*. Breeding programs for *A. auriculiformis*, *A. mangium* and its hybrid were started in the 1990s in Vietnam. Recently, research on polyploid *Acacia* has been conducted under a collaboration between the University of Tasmania (UTAS) and the Vietnamese Academy of Forest Sciences (VAFS) that aims to produce sterile and outstanding triploid (3*x*) genotypes. Molecular markers could facilitate the development of improved breeding methods, lead to better deployment programs and expand our knowledge of acacia genetics. Microsatellite markers (SSR) are among the most informative molecular markers. However, there is a limited number of SSR markers currently available for acacia, especially ones that works well across taxa. Therefore, the development of additional microsatellite markers that will function in *A. auriculiformis*, *A. mangium* and their interspecific hybrid is required for future research. The overall aim of this study was to develop such markers and show that they can be very useful to acacia breeding and deployment programs in Vietnam.

A highly informative (probability of identity= 4.1×10^{-13}) and easy to use set of 16 SSR markers was developed for acacia hybrid and their parental species. The markers were optimised for assay in four multiplex mixes and used to genotype range-wide samples of *A. mangium; A. auriculiformis,* and putative F_1 hybrids. Ten of the markers were highly polymorphic in each species and proved useful for fingerprinting, studying diversity and parentage analysis. Six of the markers were much less polymorphic and because the species did not share any alleles these provided 'species-diagnostic' markers. The six species-diagnostic markers, in combination with the statistical package HIest, enabled accurate

allocation of genotypes to the two pure species, F_1 and F_2 interspecific hybrids and backcrosses with good degree of accuracy (96%-100%). Many acacia hybrids identified using morphology in ongoing selection program in Vietnam (65 out of 160 putative hybrid clones) were found to be mistaken (most were pure *A. auriculiformis*) using the marker set.

The set of SSR markers were then applied to verify the 'purity' status of putatively pure *A. mangium* and *A. auriculiformis* clones in paired clonal seed orchards. Approximately 4% of the genotypes in these seed orchards were found to be either F_1 hybrids or backcrosses. This shows that inter-species contamination is an issue requiring management in both pure-species and hybrid breeding of these species in Vietnam. The acacia pollen dispersal pattern was also examined in these paired clonal seed orchards with a large number of open-pollinated progeny (5,400 seedlings) using a pooling strategy. The decay in the level of hybridisation with distance followed a power function with a negative exponent. There were no differences between species and no interaction between distance and species in pollen dispersal. The restriction of most F_1 hybridisation to within 100 m of species separation presents clear opportunities to manage the genetic purity of pure species seed orchard as well as to improve hybrid seed yields when that is the goal.

Breeding system and seed characteristics of induced allotetraploid acacia hybrid were compared to those of their diploid progenitors, as well as growth characteristics of their progenies. Despite the fact that peak flowering differed by two months between ploidy, there was overlap in flowering time between them, showing potential for production of triploids through open pollination. However, of the 1350 seedlings analysed none were triploid. Allotetraploid acacia hybrid produced bigger seeds than those in their diploid progenitors. The outcrossing rate of allotetraploid was very low (14%) in comparison with the diploid progenitors (87%). The inbreeding depression in growth at 12 months after planting in diploid seedlings produced by selfing (33%) was greater than that in allotetraploid (17%). By investigating the inheritance of molecular markers in outcrossed

progenies, we found evidence that the allotetraploid acacia hybrid has intermediate or mix model inheritance (with both disomic and tetrasomic marker inheritance) and it thus appears to behave as a segmental allotetraploid. Thus, breeding programs aiming to develop advanced generation allotetraploid acacias may have to select for fertility.

Molecular markers have many uses in support of acacia breeding. They can be used to detect hybrid individuals from open-pollinated seed sources and this is useful because identification using morphology is prone to misidentification. Secondly, DNA fingerprinting is a valuable tool for paternity analysis and monitoring clonal identity as well as genetic diversity and therefore has a role in monitoring controlled crossing programs, evaluating assumptions regarding pollination in seed orchards and verification of clonal material during propagation. The study also contributes new knowledge of the breeding system, reproductive and growth characteristics of allotetraploid acacia hybrid.

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Chapter 1 General introduction

1.1. Tropical acacias in forestry

Acacia is the second largest genus of the *Leguminosae* family. Taxonomic classification of *Acacia* remains a subject of considerable debate (Byrne 2002; Maslin et al. 2003; Murphy 2008). At the Nomenclature Section of the XVII International Botanical Congress (IBC) in Melbourne in 2011, a new classification splitting the old *Acacia* into five genera was approved (Maslin 2015; McNeill and Turland 2011) making *Acacia* the largest genus under this classification which now includes 1067 species/taxa that are mostly distributed in the Australia/Pacific region (1064 species) as well as Asia (13 species) (Maslin 2015). The second largest genus, *Senegalia* includes 201 species/taxa and the third, *Vachellia*, contain 163 species/taxa and species from these genera are native to the Americas, Africa, Asia and Australia. The other two small genera, *Acaciella* and *Mariosousa* include 15 and 13 species respectively, that are only found in the Americas (Maslin 2015).

Acacia is a nitrogen-fixing genus, and some *Acacia* species have been introduced in many countries due to their fast growth rate and high adaptability (Brockwell et al. 2005; Midgley and Turnbull 2003). Acacias are used for multiple purposes including pulp and paper, fuelwood, posts, sawn timber, stock fodder, human food, shade, windbreaks, cosmetics, soil and environmental protection (Brockwell et al. 2005; Griffin et al. 2011).

Three phyllodenous (i.e. lacking true leaves) species from section *Juliflorae* in the subgenus *Phyllodineae, A. auriculiformis, A. crassicarpa* and *A. mangium,* have become important plantation species in the tropics with an estimated 1.85 M ha established by 2010 (Griffin et al. 2011; Harwood et al. 2015). In Vietnam, about 1.1 M ha of tropical *Acacia* plantations had been established by 2013, contributing about 50% of the total plantation area in that

country (Nambiar et al. 2015). *A. auriculiformis* and *A. mangium*, together with their interspecific hybrid (hereafter termed acacia hybrid), are the most widely planted *Acacia* taxa in Vietnam, accounting for over 99% of *Acacia* plantations (Nambiar et al. 2015). The hybrid between *A. auriculiformis* and *A. mangium* is the only hybrid combination detected in Vietnam since *A. crassicarpa* does not hybridise with the other species (Harwood et al. 2015). The hybrids between *A. auriculiformis* and *A. mangium* will hereafter be called Acacia hybrid. This can be contrasted with *Eucalyptus*, where many interspecific hybrid combinations are found in both natural populations and introduced plantations (Griffin et al. 1988), and several interspecific hybrid combinations within the subgenus *Symphyomyrtus* are economically important in plantation forestry (Harwood 2014). Acacia hybrid has become increasingly important in Vietnam due to its excellent growth and adaptability (Kha et al. 2012); therefore, research on the reproductive biology and genetics of acacia hybrid is required to support its breeding and use.

1.1.1. *Acacia mangium*

Acacia mangium is native to Australia, Papua New Guinea (PNG) and adjacent regions of West Papua Province in Indonesia. In Australia, the natural distribution is limited to two regions of NE Queensland: (a) from Jardine River (11°20'S) to Claudie River (12°44'S) and (b) from Ayton (15°54'S) to south of Ingham (18°30'S) (Doran and Turnbull 1997) (Maps are presented in Chapter 2). *Acacia mangium* was first introduced from Australia to Sabah, Malaysia in 1966. The species was subsequently found to be suitable for plantation establishment in many tropical countries. By 2011, there were approximately 1.4 M ha of *A. mangium* plantations worldwide, mainly in SE Asia (Griffin et al. 2011), and by 2014, an estimated 0.4 M ha in Vietnam alone (Nambiar et al. 2015).

In tropical areas, *A. mangium* shows vigorous growth with mean annual wood volume

increments of up to 30 m^3 ha^{-1} per year on suitable habitats. *Acacia mangium* grows up to 30m in height and up to 90 cm in diameter (Midgley and Turnbull 2003). The wood is commonly used for pulp and paper production, furniture making, veneer and plywood. Furthermore, *A. mangium* can grow on sites with low fertility and impeded drainage (Turnbull et al. 1997) and has also been planted in reforestation programmes and for erosion control.

Flowers of *A. mangium* are white or creamy in rather loose spikes up to 10 cm long arranged singularly or in pairs. The number of flowers per spike varies from 150 to more than 200 and is higher than that of *A. auriculiformis* which has about 50 to 100 flowers per spike (Sedgley et al. 1992b). Flowering phenology differs throughout its natural and planted ranges depending on the climate conditions. In Australian natural stands, flowers are presented from February to May and the seed matures from October to December (Sedgley et al. 1992b). In Vietnam, flowering time starts from September to January and the seed are ready to harvest from late March to May (Nghiem et al. 2011). The seedlings initially produce pinnate and bipinnate compound true leaves. Approximately, 7-8 weeks after sowing, true leaves begin to be replaced by phyllodes and for several nodes, intermediate stages between phyllodes and leaves are found. Older seedlings only produce phyllodes (Fig. 1.1). The phyllodes have three to four longitudinal main veins, which join on the dorsal margin at the base of the phyllode with fine secondary veins (Awang and Taylor 1993).

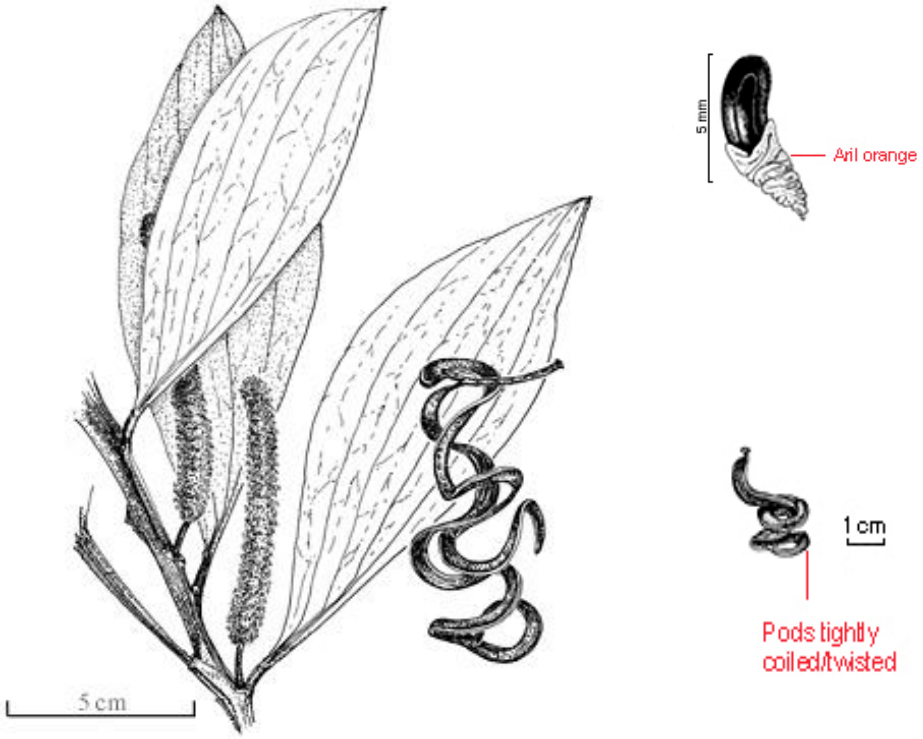


Figure 1.1. Phyllodes, flowers, pods and seed of *Acacia mangium* (adapted from http://worldwidewattle.com/speciesgallery/mangium.php)

Acacia mangium is prone to several diseases such as heart rot, root rot and phyllode rust (Lee 2004; Thu et al. 2014). *Acacia mangium* can have a high rate of heart rot, ranging from 50-98%, whereas *A. auriculiformis* is less prone to heart rot (Barry et al. 2005; Ito 2002; Ito and Nanis 1997; Mihara et al. 2005). Since 2010s, a vascular wilt and stem canker disease caused by a species of *Ceratocystis* has become the most damaging disease of *Acacia*, especially *A. mangium*, causing large-scale mortality in Indonesia and Malaysia (Brawner et al. 2015; Tarigan et al. 2011; Thu et al. 2012). By 2015, *Ceratocystis* wilt and stem canker were affecting approximately 2,000 ha of *A. mangium* plantations across Vietnam (Plant Protection Department 2015).

Acacia mangium was identified as having extremely low levels of genetic diversity using allozymes ($H_e = 0.017$) compared to other acacias (mean $H_e = 0.147$) and other forest species

(0.182 in eucalypts and 0.207 in conifers) (Moran et al. 1989a). The genetic diversity of this species was estimated to be 3-8 times higher using Restriction Fragment Length Polymorphism (RFLP) markers (Butcher et al. 1998). The level of genetic variation detected in *A. mangium* using SSR markers was three times higher than that detected using RFLP (Butcher et al. 1999), illustrating the advantages of newly developed markers for evaluating genetic variation. However, *A. mangium* is still considered to have unusually low genetic diversity levels (Butcher et al. 2004).

Butcher et al. (2004) found that *A. mangium* populations from New Guinea with high genetic diversity were completely outcrossed while small and fragmented populations had low outcrossing rates (-30%). There was no evidence of outcrossing in the Sidei (Irian Jaya) population consistent with its extremely low level of genetic diversity (Butcher et al. 2004).

1.1.2. *Acacia auriculiformis*

Acacia auriculiformis is distributed naturally in Australia, south-western PNG and adjacent West Papua, Indonesia (Turnbull 1986). In Australia, *A. auriculiformis* occurs in the north of the Northern Territory and in Cape York Peninsula (Queensland). It also occurs on islands between mainland Australia and Papua New Guinea. The natural altitudinal range of *A. auriculiformis* is from sea level to about 400 m (Pinyopusarerk 1990) (Maps are presented in Chapter 2).

Acacia auriculiformis, like *A. mangium*, is a fast-growing and evergreen tree species, which can grow up to 30 m tall and 80 cm in diameter (Pinyopusarerk 1990). *Acacia auriculiformis* was introduced to SE Asia in the 1960s and rapidly became an important forestry species in tropical areas due to its wide site adaptability and uses in short rotation (Hai et al. 2008). In 2011, there was an estimated 0.1 M ha plantation of *A. auriculiformis* in Vietnam (Griffin et al. 2011).

Acacia auriculiformis starts flowering at the age of about 18 months and can produce heavy seed crops after 4-5 years. Its flowering time is highly variable depending on site in Malaysia (Ibrahim and Awang 1992), but it typically flowers on average a little later than *A. mangium* where the two species are growing together in Vietnam (Nghiem et al. 2011). However, the two species can overlap in flowering time, thereby allowing natural hybridisation where they co-occur (Sedgley et al. 1992a). Newly germinated *A. auriculiformis* seedlings produce bipinnate leaves (within 2 weeks), then switch to phyllodes, generally at the third or fourth leaf node. The phyllodes of *A. auriculiformis* are blade-like and slightly curved, can reach up to 1.5-2.5 cm in width, and have three main veins with a marginal gland near the base. The flowers are in loose, yellow spikes at the phyllode axils or in clusters of spikes at the stem tips (Pinyopusarerk 1990) (Fig. 1.2).

Figure 1.2. Phyllodes, flowers and pods of *Acacia auriculiformis* (Source: Von Mueller, 1888)

The genetic diversity of *A. auriculiformis* (expected heterozygosity, H_e = 0.081) is higher than that of *A. mangium* (H_e = 0.017) when assessed using isozyme markers (Moran et al. 1989a; Moran et al. 1989b; Wickneswari and Norwati 1993). However, the Northern Territory populations of *A. auriculiformis* have very low diversity (He= 0.002) (Wickneswari and Norwati 1993). In its natural range, *A. auriculiformis* exhibits generally high outcrossing rates (~93%), estimated using 15 isozyme markers (Moran et al. 1989b).

1.1.3. A. mangium x *A. auriculiformis* hybrid

Naturally occurring *A. mangium* x *A. auriculiformis* hybrids were first noted in Sabah in the late 1970s, especially when *A. mangium* and *A. auriculiformis* were planted together or in close proximity (Tsai 1988). Acacia hybrids have been detected infrequently in Papua New Guinea where the species co-occur naturally (Gunn et al. 1989a).

Acacia hybrid has a morphological appearance that is intermediate between the parental species for some traits; for example, inflorescence, flowers, seeds and phyllode shape as well as the time of its development. Careful selection, cloning and testing have allowed the identification of acacia hybrid clones with enhanced properties compared to either parental species, such as vigorous growth, better stem-form, lighter branching, better self-pruning ability and smoother bark (Bowen 1981; Kha 2001; Pinso and Nasi 1992). Acacia hybrid can show increased resistance to pests and diseases compared to its parental species (Pinso and Nasi 1992). Acacia hybrid can exhibit higher pulp yield than either *A. mangium* or *A. auriculiformis*, whereas lignin content is not significantly different from the parental species (Yahya et al. 2010). In addition, the paper produced from acacia hybrid has better folding and pulling strength than paper produced from *A. mangium* or *A. auriculiformis*. Therefore, acacia hybrid is economically important for pulpwood production in Vietnam (Kha 2001).

1.1.4. Breeding programs for tropical Acacias

The initial plantations of tropical *Acacia* species were established using wild and unimproved open pollinated seeds. Domestication initially focused on identifying outstanding provenances based on their adaptation to site, growth rate and form at testing sites. Subsequently, wood properties and tolerance of pests, diseases and abiotic stresses were also considered (Harwood et al. 2015). The fastest-growing provenances were identified via provenance trials. In tropical areas, provenances from northern Queensland and PNG are favoured for *A. auriculiformis* while for *A. mangium* provenances from the Western Province of PNG and the Claudie River (far north Queensland, Australia) region showed the best growth performance. The Oriomo (PNG) provenance of *A. mangium* has shown outstanding vigour and tree form in tropical areas (Harwood et al. 2015).

In Vietnam, breeding of tropical acacias commenced in 1996. The base populations of both *A. auriculiformis* and *A. mangium* included open pollinated families from a number of fast-growing provenances and from seed orchards in Australia and Thailand. These were established as progeny trials testing over 150 families of each species. Some progeny trials were selectively thinned to create seedling seed orchards (SSOs) to produce better quality seeds for future breeding and also for plantations. Importantly, reproductive biology research, including detailed flowering and seedling development studies, commenced to support these breeding activities (Butcher et al. 2004; Harwood et al. 2004; Sedgley et al. 1992a; Sedgley et al. 1992b). Open pollinated seed from the best trees from the first-generation SSOs was collected to establish second-generation progeny trials. Clonal seed orchards (CSOs) were also established in order to increase the selection intensity and genetic gain over that attainable from SSOs (Hai et al. 2015; Harwood et al. 2015). In genetic gain trials in Vietnam, the wood volume per hectare for the best families of first-generation *A. auriculiformis* seed orchards was more than double that of a local commercial seed source

(Hai et al. 2008). Similarly, improved *A. mangium* seed sources yielded a 70% increase in volume per hectare compared to a local commercial seed source of this species (Nambiar et al. 2015).

The wide use of *Acacia* species poses special challenges of invasiveness. There are at least 23 *Acacia* species, including *A. auriculiformis* and *A. mangium*, currently considered as invasive (Blakesley et al. 2002; Griffin et al. 2011) due to their high adaptability and abundant production of long-lived seed. Development of infertile triploid clones is one possible strategy to reduce invasiveness. Polyploid breeding of *A. auriculiformis*, *A. mangium* and acacia hybrid is underway in Vietnam in order to improve adaptability and wood properties as well as to produce infertile triploid genotypes (Griffin et al. 2015). Phenology and reproductive biology research on autotetraploid *A. mangium* trees has been conducted (Nghiem et al. 2011; Nghiem et al. 2016; Nghiem et al. 2013). Polyploid breeding has produced natural and control pollinated triploid *Acacia* offspring, which are currently being evaluated under field conditions (Nghiem unpublished data).

Controlled pollination of tropical acacias is technically feasible (Nghiem et al. 2016), but it requires significant resources (cost and time) to produce more than a few crosses per year. Therefore, advancing breeding populations have relied on open pollinated seeds. For similar reasons, most candidate genotypes of acacia hybrid have been produced by open pollination. It is challenging to identify F_1 hybrids positively using morphological traits due to their wide intraspecific phenotypic variation (Rufelds 1988). Hybrids may be identified at the seed or early seedling stage (i.e. 2 weeks old) using the isozyme marker GDH-1 (Wickneswari 1989), Sequenced Characterised Amplified Region (Aggarwal et al. 2011) markers (Huang et al. 2005) or single nucleotide polymorphisms (SNPs) (Yuskianti et al. 2011b). However, to my knowledge, these markers are not used operationally for breeding purposes.

In Vietnam, the targeted development of acacia hybrid started in the early 1990s by selecting natural hybrid individuals in young *A. mangium* plantations in northern Vietnam based on their phenotypic superiority (Kha 2001). The best 60 candidates exhibiting outstanding growth and form were coppiced and propagated. Twenty-four of these clones which could be easily propagated were tested in clonal screening trials in different sites where growth, health and tree form traits were assessed. This extensive evaluation process resulted in the selection of four superior acacia hybrid clones suitable for commercial planting. Similar selection and clonal development were conducted in southern Vietnam (Kha 2001). By 2014, there were approximately 400,000 ha of clonal acacia hybrid plantations in Vietnam but only about 10 clones are planted commercially (Nambiar et al. 2015)

Hybridising orchards were set up in Vietnam for open pollinated production of new hybrid genotypes. These consisted of sets of selected *A. auriculiformis* and *A. mangium* clones in adjacent rows. One such orchard also included rows of tetraploid *A. mangium* with the aim of producing open pollinated polyploid offspring as well as diploid hybrids (Griffin et al. 2015; Harwood et al. 2015).

Over the last decade, hybrid breeding in Vietnam has involved large-scale screening of approximately 500 putative acacia hybrid genotypes selected from open pollinated pure-species seedlots using morphological criteria (Harwood et al. 2015). In addition, hundreds of hybrid genotypes produced by controlled crossing among selected pure-species parent trees have also been tested in field trials (Nghiem unpub. data). Superior candidates identified in seedling trials of hybrid genotypes have been captured as clones, propagated and re-tested in clone screening trials. These recent hybrid clone trials show many new hybrid candidates that have faster early growth than the current commercial clones (Harwood et al. 2015). These new acacia hybrid clones are also being evaluated for disease resistance and wind resistance.

1.2. Molecular markers in tree breeding programs

1.2.1. Molecular markers

Genetic markers are traits that show the genetic differences between individuals or species. In plants, there are two major types of genetic markers: (1) morphological markers (also called 'classical' or 'visible' markers and (2) molecular markers which reveal variation in DNA or enzymes (i.e. isozymes) (White et al. 2007). Markers can be categorised into co-dominant or dominant depending on the ability to detect heterozygous individuals.

The oldest type of marker are morphological markers that can easily be observed visually such as height, colour and shape. However, these markers are not as useful as molecular markers because they are usually dominant, suffer from epistatic interactions and are potentially influenced by the environment and the developmental stage of plants.

The first type of molecular marker to be used in *Acacia* (Moran et al. 1989a; Moran et al. 1989b) was enzyme markers (also called isozyme or allozyme markers). These are enzyme proteins that differ in sizes and/or charge between individuals and are detected using electrophoresis and enzyme specific staining. Isozyme markers are limited in number and their degree of polymorphism and this has limited their practical use (Schlotterer 2004).

DNA markers have largely replaced all other types of molecular markers. These are abundant and stable under different environments and plant developmental stages (White et al. 2007). These markers are usually located in non-coding regions of DNA; therefore, are considered to be selectively neutral. DNA markers are one of the most powerful genetic tools and have multiple uses including genetic diversity assessment, fingerprinting and linkage map construction (White et al. 2007). DNA markers can be classified according to how these are generated – via hybridisation or polymerase chain reaction (PCR). The main types of markers using hybridisation are Restriction Fragment Length Polymorphism (RFLP) and array-based markers. However, RFLP is little used nowadays. Arrays are usually

expensive to set up but once constructed can be used to quickly screen tens or hundreds of thousands of Single Nucleotide Polymorphisms (SNPs). There are many types of PCR based markers, e.g. Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR) or microsatellite, Sequenced Characterised Amplified Region (SCAR) (Agarwal et al. 2008; Neale et al. 1992; Neale and Kremer 2011; White et al. 2007). The advantages and disadvantages of commonly used molecular markers are summarised in Table 1.1.

Table 1.1. Comparison of molecular markers

Marker	Advantages	Disadvantages
SNPs	• Low mutation rate • The most abundant type of DNA markers • Easy to genotype • Cross-study comparisons are easy • Data repositories already exist • SNPs can be assayed via NGS, using methods that don't need prior marker development	• Substantial rate heterogeneity among sites • Expensive to isolate • Ascertainment bias • Low information content
SSR	• The most polymorphic type of marker • Low ascertainment bias • Easy to isolate	• High mutation rate • Complex mutation behaviour • Average abundance • Difficult to automate • Cross-study comparisons require special preparation
AFLP	• High abundance and informative • Considerable reproducibility • No sequence information needed	• Purified, high molecular weight DNA • Dominant markers • Technically demanding
RAPD and derivatives	• Cheap • Produces a large number of bands which can be converted to SCAR markers with difficulty	• Low reproducibility • Dominant markers • Difficult to analyse • Difficult to automate • Cross-study comparisons are difficult
SCAR	• More informative for genetic mapping • Easy, reliable and reproducible	• May require radioactive isotopes • High cost • Requires sequence information

(Source: Adapted from (Kiran et al. 2010; Schlotterer 2004)

1.2.2. Microsatellites

Microsatellites or simple sequence repeat (SSR) markers are found in every organism (Hancock 1999). They are short tandemly repeated DNA sequences, consisting of repeated units of 1 to 6bp in length. There are three most common repeat types: (1) dinucleotide repeats; (2) trinucleotide repeats; and (3) tetranucleotide repeats. Dinucleotide repeats occur most frequently in genomes and their density varies widely among species; hence, they have been used frequently to study genetic diversity. In plants, dinucleotide repeats differ between species and are rich in either TA or GA repeats (Depeiges et al. 1995). In some forest species, for example, *Eucalyptus*, GA repeats are the major dinucleotide motif (Byrne et al. 1996), while in *Pinus*, the most common repeats are AC and AG (Echt et al. 1998). The important features of microsatellites for genetic studies are that they are codominant, and can display high heterozygosity among and within individuals, populations and species. They are considered to be typically selectively neutral and randomly distributed across the entire genome (Jarne and Lagoda 1996).

A disadvantage of SSR markers is the time and cost during the development stage. Due to the investment in developing SSR markers, researchers consider that the ability to use SSR markers in related species is an economically efficient method to enrich SSR marker databases (Peakall et al. 1998). This has been successfully implemented in *Eucalyptus*, where the transfer of microsatellite markers among related species has been demonstrated (Butcher et al. 2008; Byrne et al. 1996; Nevill et al. 2008; Steane et al. 2001). However, transferability of SSR markers between species, genera and families is not always successful, as evidenced in *Acacia* (Adamski et al. 2013; Butcher et al. 2000a; Guillemaud et al. 2015; Omondi et al. 2015; Omondi et al. 2010). In Chapter 2 of this thesis, the development of SSR markers which are informative in acacia hybrid and its parental species will be reported.

1.2.3. Application of microsatellite markers in forest tree genetics

In 1994, Smith and Devey (1994) developed the first microsatellites in a forest tree species, *Pinus radiata*. These markers showed a high level of polymorphism with an average of six alleles detected per marker, with an observed heterozygosity (H_o) of 0.6 – 0.65. Subsequently, SSR markers were developed in many other forest tree species including: *Pinus* (Echt et al. 1998; Karhu et al. 2000), *Quercus* (oak) (Dow et al. 1995), *Eucalyptus* (Butcher et al. 2008; Byrne et al. 1996; Payn et al. 2008; Steane et al. 2011; Steane et al. 2001), *Acacia* (Butcher et al. 2000a; Guillemaud et al. 2015; Omondi et al. 2015) and some tropical forest species (Chase et al. 1996). Microsatellites can be used for various purposes, including (1) genetic diversity assessment, (2) genotype verification, (3) mating (breeding) system analysis, (4) taxonomic studies, and (5) linkage mapping and QTL analysis.

An important application of microsatellite markers is the study of population genetic structure and genetic diversity (Agarwal et al. 2008; Butcher et al. 1999; Neale et al. 1992; Wang 2001; White et al. 2007), especially as microsatellites provide greater resolution than other markers for populations with low genetic diversity. Analysis of the genetic structure of forest tree species gives insight into the ongoing process of genetic differentiation among populations and the effect of selection on genetic diversity in breeding populations (Wang 2001). In forestry, SSR markers have been used to evaluate genetic diversity of numerous species including teak *(Tectona grandis)* (Alcântara and Veasey 2013; Fofana et al. 2009; Hansen et al. 2015; Huang et al. 2016; Minn et al. 2014) and *Eucalyptus* (Cupertino et al. 2011; de Souza et al. 2010; Jones et al. 2006; Santos et al. 2011). A total of 25 SSR markers were used to evaluate the genetic diversity in an *A. mangium* SSO in Wonogiri (Indonesia) (Yuskianti and Isoda 2012). In this orchard, PNG provenances were shown to have higher genetic diversity than Queensland provenances. In a study of naturally occurring diploid and tetraploid *Senegalia senegal* (previously *Acacia senegal*), the genetic diversity of the

diploid population was higher than the tetraploid population (Assoumane et al. 2013). Importantly these results supported the utility of SSR markers in genetic studies of *Acacia* spp.

The power of microsatellite markers to genetically discriminate between individuals or groups of individuals is typically high. Specifically, in forestry, these markers are widely used to characterise germplasm and for individual identification, e.g. *Eucalyptus* (Kirst et al. 2005; Sumathi and Yasodha 2014), *Populus* (Ciftci et al. 2017; Liesebach et al. 2010) and teak (*Tectona grandis)* (Huang et al. 2016). Microsatellites have been used to verify genotypes of control pollinated triploid offspring of acacia hybrid (Nghiem et al. 2016). Microsatellite markers were also used to detect polyploid *S. senegal* individuals in natural populations and to estimate the outcrossing rate of the diploid natural population. The relationship within and between diploid and polyploid families was also assessed in order to support breeding activities for this species (Diallo et al. 2015).

In seed orchard management, information on outcrossing rates, pollen dispersal and pollen contamination is critical to establish the quality of the seed. Microsatellite markers have been widely used for estimating mating system parameters and pollen contamination in seed orchards of various forest tree species including acacia taxa. In Vietnam, the outcrossing rates in six different seed orchards of *A. mangium* were estimated using six microsatellite markers (Butcher et al. 2004). These researchers found that four seed orchards had high outcrossing rates, one showed 49% outcrossing, whereas the sixth seed orchard had low outcrossing (13%). The high degree of selfing rate in this orchard was associated with a low proportion of flowering trees and low intensity of flowering. A follow-up study by Harwood et al. (2004) showed that selfed progeny grew more slowly than outcrossed progeny indicating inbreeding depression for growth, which is common in tree species (Del Castillo and Trujillo 2008). Microsatellite markers were also used to compare the outcrossing rates

of diploid and autotetraploid *A. mangium*. The results showed that diploids were predominantly outcrossing (97%), whereas tetraploids yielded only 2% outcrossed progeny (Griffin et al. 2012).

The mating pattern of a *Pinus merkusii* SSO evaluated using SSR markers (Nurtjahjaningsih et al. 2007) found random mating system in this orchard (in the sense that all trees performed equally in genetic exchange), with some pollen contamination from a nearby plantation. In a similar study of a *P. brutia* orchard, pollen gene flow from trees in a surrounding stand reached up to 85.7% (Kaya et al. 2006). Contamination of eucalypt seed orchards from nearby plantations have also been found (Rao et al. 2008). Gene flow among cultivated poplars and black poplar was identified by using SSR markers in combination with isozyme markers (Vanden Broeck et al. 2004). A high level of genetic contamination from nearby natural stands and plantations (25.5 - 32%) was also detected in an introduced stand of *A. saligna* in Western Australia (Millar et al. 2012). Contamination of seed orchards from non-orchard pollen leads to loss of gains from breeding in plantations deployed from this seed.

Breeding acacia involves quantitative traits, such as wood yield, wood quality, or pulp yield, are usually controlled by many genes. Some regions around these genes can be identified by quantitative trait loci (QTL) analysis. By using molecular markers closely linked to, or located within one or more QTL, information at the DNA level can be used for early selection. Microsatellites have been used to construct genetic linkage maps in some commercial forest species such as *Eucalyptus grandis* and *E. urophylla* (Brondani et al. 2002; Brondani et al. 1998), *Pinus radiata* and *P. taeda* (Devey et al. 2004; Devey et al. 1999) and *Eucalyptus globulus* (Freeman 2006) in combination with other markers (e.g., isozyme, AFLP, RFLPs and RAPDs). Several QTLs for wood quality traits such as cellulose content have been detected in *Eucalyptus* (Freeman et al. 2013; Grattapaglia et al. 2012) with their estimated effects ranging from 5% to 15%. In *A. mangium*, genetic linkage maps were

created (Butcher and Moran 2000; Butcher et al. 2000b) using 219 RFLP and 33 SSR markers, providing a reference map in *Acacia*.

1.3. Research questions and objectives

Molecular markers could improve the management of tropical acacia breeding programs in several ways. For example, (1) monitoring genetic diversity of breeding populations to prevent inbreeding (Yuskianti and Isoda 2012), (2) calculating genetic distances among selections in order to improve crossing decisions (to maximise genetic diversity and reduce inbreeding), (3) investigating breeding systems in diploid and polyploid *Acacia* seed orchards to maximise seed quality for deployment, (4) identifying hybrids among open pollinated progeny and (5) checking levels of contamination in pure species breeding populations and seed orchards of *A. mangium* and *A. auriculiformis*.

When the taxonomic status is deduced from morphological criteria, errors in identification of pure species and hybrid genotypes can be costly. For example, hybrid individuals in pure-species trials of *A. auriculiformis* and *A. mangium* may be selected by mistake because of their vigorous growth, and pure-species breeding populations can become increasingly contaminated, leading to the incorrect calculation of genetic parameters and genetic rankings in the population. Similarly, incorrect identification of non-hybrid genotypes and their inclusion in hybrid clonal testing programs results in wasted effort and waste of scarce experimental land for testing, and a reduction in the size of the population of true hybrid genotypes under testing. The ability to accurately identify the taxonomic status of an individual (*A. auriculiformis*, *A. mangium* or various types of hybrids including F_1 hybrids and backcrosses) using molecular markers could thus help increase gains from breeding programs and reduce waste. Since the cost of molecular analysis is reducing, making use of molecular tools to support breeding becomes increasingly attractive.

This thesis aims to (1) develop a highly informative and easy to use set of SSR markers that work well with acacia hybrid and its parental species, and (2) demonstrate applications of the SSR markers in support of the acacia breeding programs in Vietnam.

Specific research questions addressed in the thesis:

- Can a diverse set of molecular markers be developed to adequately assess genetic diversity within and discriminate between Vietnam's two main *Acacia* species used in plantation, *A. auriculiformis* and *A. mangium* and their hybrid?
- Can the markers uniquely identify individual clones for checking identity in clonal development and clonal forestry?
- Can these molecular markers be applied to check the extent of hybridity in Vietnam's pure-species and hybrid breeding populations?
- Can these molecular markers be used to study breeding systems in both diploid and polyploid breeding populations of acacia hybrid?
- Can the markers improve our understanding of the production of hybrid genotypes under open pollination?

The thesis concludes by reviewing promising applications of these SSR markers for *Acacia* breeding in Vietnam and considering how their use can be best integrated into practical breeding programs.

Chapter 2 A multiplexed set of microsatellite markers for discriminating *Acacia mangium, A. auriculiformis* and their hybrid

2.1. Abstract

In order to assist breeding and gene pool conservation in tropical Acacias we aimed to develop a set of multipurpose SSR markers for use in both *A. mangium* and *A. auriculiformis*. A total of 51 SSR markers (developed in *A. mangium* and natural *A. mangium x A. auriculiformis* hybrid) were tested. A final set of 16 well performing SSR markers were identified, six of which were species diagnostic. The markers were optimised for assay in four multiplex mixes and used to genotype range-wide samples of *A. mangium*, *A. auriculiformis*, and putative F_1 hybrids. Simulation analysis was used to investigate the power of the markers for identifying the pure species and their F_1, F_2 and backcross hybrids. The six species diagnostic markers were particularly powerful for detecting F_1 hybrids from pure species but could also discriminate the pure species from F_2 and backcross progenies in most cases (97%). STRUCTURE analysis using all 16 markers was likewise able to distinguish these cross types and pure species sets. Both sets of markers had difficulties in distinguishing F_2 and backcross progenies. However, identifying F_1 from pure species is the current primary concern in countries where these species are planted. The SSR marker set also has direct application in DNA profiling (probability of identity = 4.1×10^{-13}), breeding system analysis and population genetics.

2.2. Introduction

Tropical *Acacia* species native to Northern Australia and New Guinea are now planted widely for forestry, with over 2 million ha of plantations worldwide (Griffin et al. 2011; Nambiar et al. 2015). Acacia plantations yield pulpwood and sawn timber and can provide environmental protection and improve soil fertility (Nambiar et al. 2015). The main species planted are *A. mangium*, *A. auriculiformis*, *A. crassicarpa* and clones of the natural hybrid between *A. mangium* and *A. auriculiformis*, here referred to as acacia hybrid. Acacia hybrid is important for wood production because of its high growth rate, and wide adaptability to various environments (Kha 2001) .

DNA markers such as microsatellites are widely used as a research tool to support tree breeding. DNA markers are simply inherited, stable under different environments and plant developmental stages. They arise from different classes of DNA mutations and DNA replication errors (Schlotterer 2004). The power of DNA markers lies in their polymorphism, giving them the ability to discriminate between individuals and groups of individuals (Anderson and Thompson 2002; Kirst et al. 2005). DNA markers can be used in forestry for monitoring clonal identity and paternity analysis and are therefore informative in controlled crossing programs, and evaluating assumptions regarding pollination in seed orchards (Neale et al. 1992). For example, Butcher et al. (2004) used six microsatellite loci to demonstrate high levels of variation in outcrossing rates between six seed orchards of *A. mangium* in Vietnam. One orchard was fully outcrossing, three had high outcrossing rates, one had about 50% outcrossing; but the sixth had a very low outcrossing rate (13%). It was found that the differences were related to flowering patterns in the orchards (Butcher et al. 2004). Microsatellite markers can also help improve our understanding of genetic resources by helping manage inbreeding in small populations (White et al. 2007) and through investigations of the level of genetic variation and genetic differentiation between and within

populations (Porth and El-Kassaby 2014). Microsatellites are also a popular molecular marker in studies aiming to identify interspecific hybrids in both plant and animal systems (Khosravi et al. 2013; Ma et al. 2014; Randi 2008; Vaha and Primmer 2006). In forestry research, microsatellites have been used for detecting hybrids in oaks (Burgarella et al. 2009), eucalypts (Larcombe et al. 2014), and poplars (Vanden Broeck et al. 2004).

DNA markers could improve the management of acacia breeding programs, by enabling more efficiency in identification of interspecific hybrids. Hybrid identification could also be important for studying contamination between pure species breeding populations of *A. mangium*, *A. auriculiformis* and acacia hybrid plantations nearby (Harwood et al. 2015). These two species hybridise easily; their hybrids are found in natural populations and in plantations (Gunn et al. 1989b; Kha 2001; Wickneswari and Norwati 1992) and it is possible for contamination between pure and F_1 breeding populations and nearby plantations to create other hybrid generations such as F_2 and backcross (Harwood et al. 2015). Detection of hybrids based on morphological traits is unreliable due to the variable morphology of *A. auriculiformis* phyllodes (Widyatmoko and Shiraishi 2003) and DNA markers offer the best prospect for more accurately determining the extent of hybridisation and backcrossing among different breeding populations. Some markers have been developed for acacia hybrid identification, for example the isozyme marker *Gdh-1* (Wickneswari 1989), RAPD markers (Wang and Hu 1996) and SCAR markers (Kato et al. 2014). Wong et al. (2012) and Sukganah et al. (2013) isolated polymorphic SNP markers in *A. auriculiformis* and *A. mangium*. However, those were not shown to be species-specific and compared to SNP markers, SSR markers offers several advantages, for example, multi-allelism and relative ease of use. Up until now, no species-diagnostics microsatellite markers have been described that discriminate between *A. mangium*, *A. auriculiformis*, and their hybrids. In addition, the cross transferability of markers between tropical acacias species has been low in previous

studies (Adamski et al. 2013; Butcher et al. 2000a). Therefore, developing a more effective set of microsatellite markers to support the breeding of *A. auriculiformis*, *A. mangium* and their interspecific hybrid is necessary.

The objective of this study was to identify a set of SSR markers that can be used for multiple purposes in pure and hybrid populations of *A. mangium and A. auriculiformis.* A useful marker set should be able to identify pure species from their F_1 interspecific hybrids, and possibly other hybrid generations (F_2 and backcross). The markers should also have applications in clonal identification, verification of progeny from controlled crossing (Nghiem et al. 2013), evaluation of breeding system in breeding populations and seed orchards (Griffin et al. 2015) as well as in gene pool conservation.

2.3. Materials and methods

2.3.1. Plant material and DNA isolation

DNA extracts from range-wide collections were provided by the Plant Genetic Laboratory, Faculty of Science and Technology (Universiti Kebangsaan Malaysia -UKM). We used 100 trees representing 20 natural provenances of *A. auriculiformis* and 130 trees representing 26 natural provenances of *A. mangium*. Five trees per provenance were used in each species. Details of the *A. auriculiformis* and *A. mangium* provenances assayed are given in Figure 2.1 and Appendix 2.1. Additionally, to evaluate the ability of the markers to detect F_1 hybrids between *A. mangium* and *A. auriculiformis*, phyllodes were collected from 50 F_1 hybrid progenies derived from 20 controlled crosses between seven *A. auriculiformis* and nine *A. mangium* parents conducted in Vietnam as part of the VAFS breeding program (see Appendix 2.2). Phyllodes were collected in the nursery or in the field, dried with silica gel (or overnight in an oven at 50^0C) and stored at room temperature before DNA isolation. DNA was extracted using DNeasy® Plant Mini Kit (Hilden, Germany), where 50 mg of

dried phyllode tissue was crushed to a powder in liquid nitrogen and then the standard Qiagen protocol was used for DNA extraction (www.qiagen.com/handbooks). DNA concentration and purity were assessed using gel electrophoresis and comparison with Lambda *Hin*d III molecular weight standard.

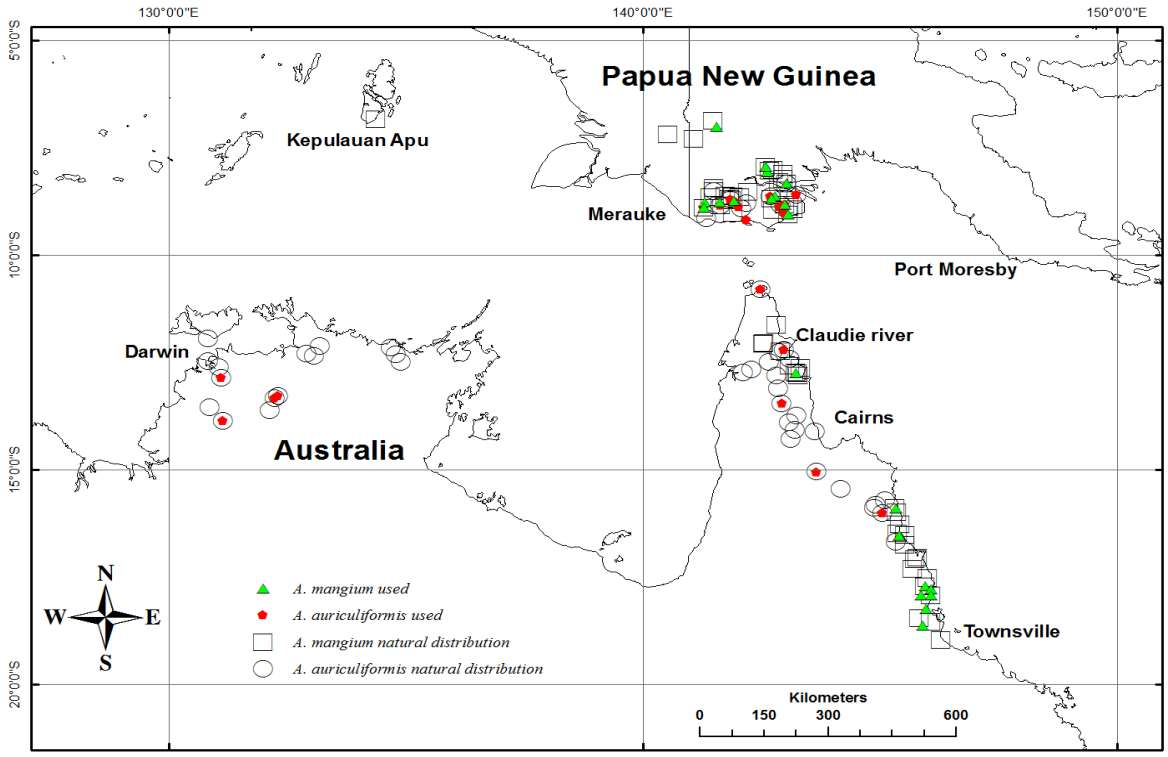


Figure 2.1. Map showing the locations of the provenances of *A. auriculiformis* and *A. mangium* sampled in this study and other natural provenances collected by CSIRO's Australian Tree Seed Centre (D. Bush, pers. comm. 2015).

2.3.2. PCR condition and PCR product analysis

Fifty-one markers were initially screened, including; five SSR markers developed by Butcher et al. (2000a) for *A. mangium* and the total of 46 SSR markers for acacia hybrid containing 15 genomic SSR markers by Ng et al. (2005), 20 EST (expressed sequence tag) SSR markers by Aggrawal et al. (2011) (Appendix 2.3) as well as 11 newly developed EST-

SSR markers. The new markers were developed from an EST database containing a total of 6,415 non-redundant ESTs from *A. auriculiformis* and *A. mangium* hybrid (Yong et al. 2011) using SSR Finder (http://61.50.158/molecularbreeding/index.jsp) to detect the presence of SSRs within ESTs. All 51 markers were first screened for cross species amplification with 16 individuals each of *A. mangium* and *A. auriculiformis*. PCRs were performed in a GeneAmp PCR system 9700 (Applied Biosystems, USA) or an Eppendorf Mastercycler EP Gradient S Thermal Cycler with a final volume of 12.5 µl, consisting of 1x PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.2 µM of each forward and reverse primer, 0.5U Taq DNA polymerase (Invitrogen, Massachusetts, USA) and 20 ng of genomic DNA. Thermocycler conditions followed Butcher et al. (2000a) with annealing temperature (Mt) ranging from 50^0C to 60^0C depending on each marker (see Appendix 2.3). PCR products were separated on 7% denaturing polyacrylamide gel following methods described by Benbouza et al. (2006).

In a second screening stage, the 16 best SSR loci, those that reliably amplified products of expected size in both species from the first screening, were tagged with fluorescent dyes NED, 6-FAM, HEX and ROX on their forward primers and PCR was conducted for all 230 samples. The 16 pairs of SSR primers were multiplexed in four mixes (see Appendix 2.4). PCR products were separated using an ABI 3730 DNA Analyzer (Applied Biosystems, USA) by 1st BASE company (www.base-asia.com); raw data were analysed using GeneMapper 3.7 (ABI, USA) software to score genotypes.

2.3.3. Data analysis

Population genetic parameters for each marker and species (N_a = Number of different alleles, H_o = Observed heterozygosity, H_e = Expected heterozygosity, F = Fixation index) were estimated (Table 2.1) using GenAlEx V6.5 (Peakall and Smouse 2012). Twenty samples

of each species were PCRed twice, and the subsequent repeated genotypes were compared at each locus to calculate the repeatability (A_0) of each marker. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between loci were tested using GENEPOP (Raymond and Rousset 1995; Rousset 2008). MICROCHECKER (Van Oosterhout et al. 2004) was also used to calculate the frequency of null alleles. Markers performances were also evaluated using the Probability of Identity for unrelated individuals (PI) and for siblings (PIsibs) (Waits et al. 2001) calculated by GenAlExV6.5 (Peakall and Smouse 2012)

In order to assess the ability of the markers to differentiate between the pure species and the F_1 individuals we used STRUCTURE (Pritchard et al. 2000) using the admixture model without *a priori* species information. A burn-in of 100,000 Markov Chain Monte Carlo (MCMC) iterations was performed with a subsequent 100,000 data generating iterations. A range of cluster number (K) from one to ten was used with 10 replicates. Evanno's delta K approach (Evanno et al. 2005) was used to determine the most appropriate number of genetic clusters (K).

2.3.4. Calculating detection power of diagnostics markers with simulated and pedigreed hybrids

HYBRIDLAB (Nielsen et al. 2006) was used to generate four hybrid populations: F_1, F_2, and backcross to each species (BC_{Aa}; BC_{Am}), as well as simulated pure species populations. These simulations were based on the allele frequencies of 127 genotypes of *A. mangium* and 96 genotypes of *A. auriculiformis* after removing seven samples that were potentially misclassified as identified in the STRUCTURE outputs. Five hundred individuals were simulated for each population. The 3,000 simulated individuals were analysed in STRUCTURE (Pritchard et al. 2000) using an *a priori* hypothesis that K=2, and the same analysis parameters as in 2.3.3. Fifty simulated samples for each category were run for

presentation of Figure 2.3. To identify pure individuals, we used a q_{value} (genotype membership) cut-off of 0.1; this value is recommended to separate hybrids from pure parental species when high identification efficiency is required (Vaha and Primmer 2006). This means if a sample has $q_{value} > 0.9$ in one cluster, the individual was deemed indistinguishable from the pure parental species. F_1 hybrids were those individuals with $q_{value} = 0.5 \pm 0.1$, and for first generation backcross were those with $q_{value} = 0.75 \pm 0.15$.

2.4. Results

2.4.1. Cross-species amplification of microsatellite markers

There were variations in amplification success between the two species for many markers. From 51 markers screened initially, 16 showed consistent high-quality cross-species amplification. Of the failed 35 markers, six were only amplified in either *A. mangium* or *A. auriculiformis*, 23 were not consistent in their amplification across both species and six gave many additional PCR products.

In the second screening stage, 100 samples of *A. auriculiformis* and 130 of *A. mangium* were amplified with the 16 selected loci. The characteristics of these 16 markers are summarised in Table 2.1. A total of 137 different alleles were found in *A. auriculiformis* compared to 151 in *A. mangium*. There was 2.6% missing data overall and the average repeatability of the marker (A_0) across both species was 93.2% (Table 2.1). The number of alleles (N_a) per marker ranged from 1 to 23 in *A. auriculiformis* and from 1 to 29 in *A. mangium*, while the expected heterozygosity (H_e) values ranged from 0 to 0.90 in *A. auriculiformis* and from 0 to 0.92 in *A. mangium*. There was no evidence of linkage disequilibrium between loci (data not shown) and null alleles had low frequencies, on average 8% in each species (Table 2.1).

The results also showed that microsatellite markers developed from genomic DNA by Butcher (Butcher et al.) (Am041, Am465 and Am387) showed more diversity (average number of alleles per loci was 16.3 for *A. auriculiformis* and 19.3 for *A. mangium*) than other markers developed and isolated from EST sequencing (Aggarwal et al. 2011; Ng et al. 2005) (average number of alleles per locus = 6.8 in both species (Table 2.1)).

Table 2.1. Characteristics of 16 markers giving consistent high-quality PCR amplification in two *Acacia* species

Marker	*A. auriculiformis*						*A. mangium*					
	N_a	H_o	H_e	F	A_0	N_u	N_a	H_o	H_e	F	A_0	N_u
AH08	2	0.16	0.19	0.16	1.00	0.12	3	0.18	0.21	0.14	1.00	0.19
AH16	20	0.64	0.9	0.19	0.90	0.04	21	0.65	0.89	0.27	0.90	0.04
AH29	10	0.81	0.86	0.06	0.90	0.03	10	0.68	0.78	0.13	0.90	0.00
AH54	2	0.34	0.44	0.23	1.00	0.08	2	0.21	0.47	0.55	1.00	0.18
AH69	4	0.55	0.67	0.18	0.90	0.16	5	0.5	0.67	0.25	0.95	0.12
AH76	7	0.61	0.71	0.14	0.90	0.07	8	0.26	0.32	0.19	0.90	0.07
AH3_6	1	0	0		0.95	0.00	1	0	0		1.00	0.00
AH3_17	3	0.25	0.36	0.31	0.90	0.13	3	0.12	0.14	0.14	0.95	0.11
PCT4&ancp8a	3	0.21	0.25	0.16	0.90	0.08	6	0.62	0.65	0.05	0.90	0.03
ancp16a&ancp17s	2	0.38	0.49	0.22	0.95	0.12	4	0.29	0.4	0.28	0.90	0.12
ancp29s&ancp30a	13	0.68	0.85	0.20	0.95	0.05	10	0.78	0.79	0.01	0.90	0.11
ancp54a&ancp55s	3	0.47	0.52	0.10	1.00	0.11	2	0.32	0.41	0.22	1.00	0.12
ancp69a&ancp70s	19	0.79	0.86	0.08	0.90	0.03	14	0.7	0.8	0.13	0.90	0.04
Am041	23	0.84	0.87	0.03	0.90	0.00	29	0.89	0.92	0.03	0.90	0.01
Am387	12	0.59	0.83	0.29	0.90	0.09	11	0.34	0.59	0.42	0.90	0.08
Am465	14	0.6	0.83	0.28	0.90	0.12	18	0.73	0.87	0.16	0.95	0.04
Mean	8.63	0.50	0.60	0.18	0.93	0.08	9.19	0.45	0.56	0.20	0.93	0.08

(N_a = Number of different alleles, H_o = Observed heterozygosity, H_e = Expected heterozygosity, F = Fixation index, A_o = Repeatability, N_u = Null Alleles frequency)

2.4.2. Marker performance

Our results confirm the effectiveness of the DNA marker set for genetic studies of both *A. mangium* and *A. auriculiformis* as well as their interspecific hybrid. The probability of identity (PI, the probability of two independent samples having the same identical genotype (Waits et al. 2001)), using all 16 microsatellites, resulted in values as low as 4.1×10^{-13} for *A. mangium*, 1.5×10^{-14} for *A. auriculiformis* and 8.4×10^{-18} in the combined dataset with both pure species and their hybrids (Table 2.2). This means it is very unlikely that two unrelated samples will have the same genotype. However, the probability of identity between related individuals (PIsibs) is not as low, being 2.6×10^{-5}, 9.8×10^{-6} and 5.7×10^{-7} for the pure *A. mangium*, *A. auriculiformis* and, the combination of these two species and their F_1 hybrids, respectively.

Table 2.2. Summary of 16 loci combination power of discrimination

Populations	PI	PIsibs
A. mangium	4.1×10^{-13}	2.6×10^{-5}
A. auriculiformis	1.5×10^{-14}	9.8×10^{-6}
A. mangium, *A. auriculiformis* and putative F1	8.4×10^{-18}	5.7×10^{-7}

(PI: the probability of two independent samples having the same identical genotype, PIsibs: the probability of identity between related individuals (Waits et al. 2001))

Six markers were species diagnostic, that is, completely independent sets of alleles were found in each species at these loci (i.e. no shared alleles) (Table 2.3). Only one of these markers (AH3_6) had a single allele per species, the other markers had between 2-4 alleles per locus in each species. A validation of these species diagnostic markers, was carried out

by screening unrelated F_1 from control pollinated crosses (Table 2.3). All 50 F_1s were heterozygous for the species-diagnostic alleles.

Table 2.3. Allele frequencies of six species diagnostic markers in two *Acacia* species and their hybrids

Marker	Allele (bp)	*A. auriculiformis* (n=100)	*A. mangium* (n=130)	Hybrid (n=50)
ancp16a&ancp17s				
	93	0.63	0.00	0.28
	95	0.37	0.00	0.22
	97	0.00	0.16	
	99	0.00	0.23	
	109	0.00	0.22	0.12
	111	0.00	0.39	0.38
AH3_6				
	217	1.00	0.00	0.50
	221	0.00	1.00	0.50
AH08				
	88	0.35	0.00	
	92	0.65	0.00	0.50
	94	0.00	0.63	0.38
	96	0.00	0.27	0.12
	98	0.00	0.10	
ancp54a&ancp55s				
	71	0.00	0.54	0.50
	73	0.00	0.42	
	79	0.59	0.00	0.39
	81	0.16	0.00	
	83	0.25	0.00	0.11
AH3_17				
	176	0.00	0.26	
	182	0.00	0.73	0.50
	184	0.19	0.00	
	186	0.64	0.00	0.50
	190	0.17	0.00	
AH54				
	67	0.00	0.71	0.35
	71	0.00	0.29	0.15
	73	0.63	0.00	0.35
	77	0.37	0.00	0.15

The optimum number of clusters (K) from STRUCTURE analysis of the pure *A. mangium*, *A. auriculiformis* and their F_1 hybrids was two (K=2) (see Appendix 2.5). The samples classified as *A. auriculiformis* were assigned genotype membership almost exclusively to cluster I except one sample that was assigned to cluster II (q_{value} < 0.9 for cluster I), and three other individuals that had q_{value} between 0.7 - 0.8. Almost all *A. mangium* samples were assigned to cluster II, except for three individuals that were assigned to cluster I (Fig.2.2). All control pollinated F_1 samples were around 50% genotype membership to cluster I with q_{value} ranging from 0.495 to 0.504.

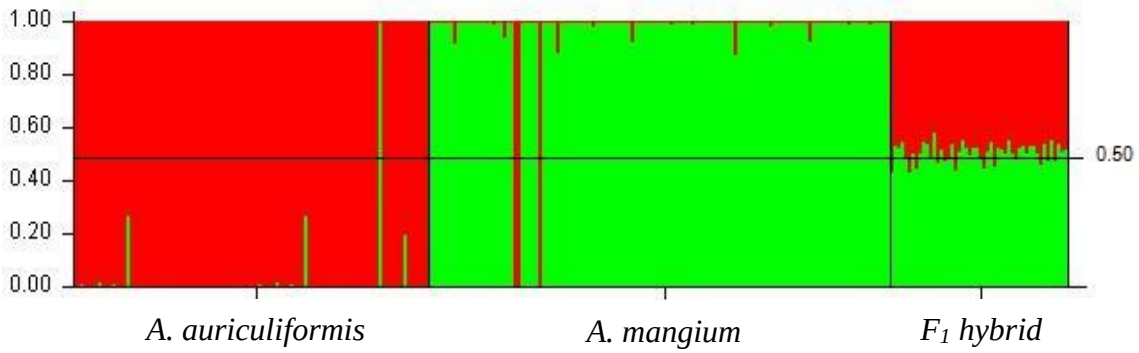


Figure 2.2. Proportion of genotype membership of pure *A. auriculiformis*, *A. mangium* and their control pollinated F_1 based on Bayesian cluster analysis using 16 molecular markers.

The six diagnostic markers give the ability to distinguish the pure species and F_1 from each other. We can use simple probability to calculate the capacity of this marker set to distinguish other hybrid generations such as F_2 and first generation backcross. The probability that an F_2 progeny will be fixed for all six diagnostic markers and be indistinguishable from one or the other pure species is 0.000488 ($(p^2)^n$ x 2, where p^2 is the probability of an F_2 being homozygous at one of n loci). The probability of a first generation

backcross sample (either BC_{Aa} and BC_{Am}) being indistinguishable from a pure species is 0.000244 ($2pq^n$) and the probability of an F_1 being indistinguishable from an F_2 or BC progeny is also 0.000244 ($2pq^n$). However, distinguishing F_2 from backcross progenies is harder and we thus investigated the ability of STRUCTURE to distinguish different hybrid and pure populations using the full set of 16 markers with simulated populations. The simulated pure, F_1, F_2, BC_{Aa} and BC_{Am} populations were all run together in STRUCTURE at K=2 (Fig. 2.3) and assigment accuracy checked using q_{value} cut-offs.

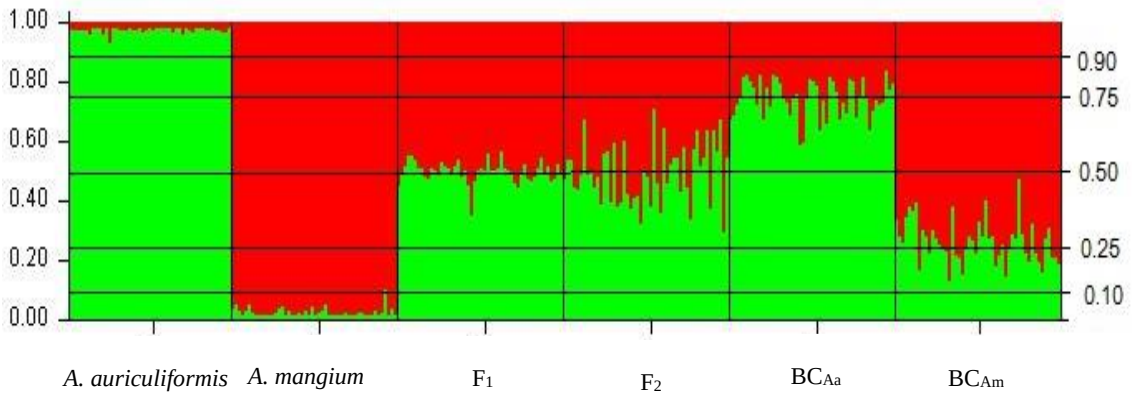


Figure 2.3. Proportion of genotype membership simulated population (n=50) of *A. auriculiformis*, *A. mangium* and their simulated hybrid generations based on Bayesian cluster analysis using 16 molecular markers

The assignment accuracy of pure species and simulated F_1 was high, reaching 100% and 98% respectively (Table 2.4), but for the backcross generations it was a little lower at 86%. As expected, distinguishing between F_1's and F_2's using STRUCTURE at the individual level is not possible, because both are expected theoretically to possesses around 50% of alleles from each parent population.

Table 2.4. Average proportion of membership (q_{value}) from STRUCTURE of each simulated population to each of the two genetic clusters and the overall accuracy of assignment (n = 500 for each population) of various *Acacia* populations

	q_{value}		Assignment accuracy
Simulated populations	*A. auriculiformis*	*A. mangium*	(%)
A. auriculiformis	0.937	0.063	100
A. mangium	0.063	0.937	100
F_1	0.504	0.496	98
F_2	0.497	0.503	--
BC_{Aa}	0.738	0.262	86
BC_{Am}	0.283	0.717	86

The six species diagnostic markers should allow discrimination of F_1 and F_2 from pure species as shown above. However, as found using the six diagnostic markers, the STRUCTURE analysis using the full set of 16 markers did not allow easy discrimination between F_2 and backcross progenies.

2.5. Discussion

By screening a total of 51 markers, we have successfully identified a set of 16 DNA markers that will have multiple applications in pure and hybrid acacia populations. The marker set includes both species diagnostic and highly polymorphic markers that can be used for population genetics, breeding system analysis, and fingerprinting in mixed and pure populations of *A. mangium* and *A. auriculiformis.* This marker set can also be used to investigate the degree of contamination in breeding populations in situations where *A. auriculiformis*, *A. mangium* and their hybrid are growing and being bred together in the same landscape, for example in Vietnam (Harwood et al. 2015). There are no other *Acacia* species planted commercially in such exotic environments which hybridise with the two species studied here (Butcher et al. 2000a; Griffin et al. 2011; Harwood et al. 2015). This means

that acacia is a simpler and more straightforward system than *Eucalyptus*. In *Eucalyptus* several widely planted species co-occur in exotic plantation environments in tropical and subtropical environments (Faria et al. 2010), which can often hybridise and microsatellite markers commonly cross-amplify in the different species. For example, within the subgenus *Symphyomyrtus*, *E. urophylla*, *E. grandis*, *E. camaldulensis*, *E. tereticornis* can co-occur and cross amplification and allele sharing among species makes identification of hybridisation problematic (Grattapaglia et al. 2012). Therefore, the species-specific SSR markers identified here in acacia will allow relatively easy discrimination of species and hybrids in countries such as Vietnam, Malaysia, Indonesia, Laos, Cambodia and China.

2.5.1. Population genetics

The approach we have taken for developing cross-species markers is to assess the transferability of markers from *A. mangium* and acacia hybrid to *A. auriculiformis*. In all, ten highly polymorphic SSR, and six species diagnostic markers (diagnostic markers) were identified. The allele diversity reached to 8.6 alleles/locus, this value is mid-range compared to other studies, being higher than *Pinus merkussi* (4.8 alleles/locus) (Nurtjahjaningsih et al.); to *A. mangium* (7.7-8.8 alleles/locus) (Yuskianti and Isoda 2012) and *A. saligna* (9.4 alleles/locus) (Millar et al. 2008); and lower than in *Eucalyptus grandis* (19.8 alleles/locus) (Kirst et al. 2005) and *Eucalyptus* in general (between 5 and 20) (Nevill et al. 2008).

Some of these markers have previously proven useful for population genetic analysis and linkage mapping studies in *A. mangium* (Butcher et al. 2000a; Butcher et al. 2004; Butcher and Moran 2000). Therefore, they should be useful for similar studies on *A. auriculiformis*. The sampling strategy applied in this study was not designed for population genetics, but the relatively high levels of genetic diversity estimated for most populations show the potential utility of the markers for detailed population level studies of *A. auriculiformis*. The high

levels of polymorphism found are in contrast to other studies that have attempted to transfer markers to *A. auriculiformis*. For example, Adamski et al. (2013) transferred 14 genomic SSR makers from *A. koa* to seven acacia species including *A. auriculiformis* and *A. mangium*, but only found 1.5 alleles per locus on average. The wide range of natural distribution samples used in the present study and the relatively narrow difference in evolutionary distances between *A. auriculiformis* and *A. mangium* in comparison with the species used in Adamski's study (Maslin et al. 2003; Maslin and Stirton 1997) are possible explanations for this finding. The set of 16 SSR markers reported here can be used to answer questions related to studies of genetic variation in natural populations and management of genetic variation in breeding populations in order to improve their genetic diversity.

A commonly used metric for measuring the power of a marker set for distinguishing between individuals is the PI (Waits et al. 2001). This measure expresses the likelihood of finding two individuals with the same multi-locus genotype in the population. In wildlife forensic cases, a PI value of between 0.001–0.0001 (depending on population size) has been used for proof of identity (Waits et al. 2001). The marker set evaluated in the present study showed a very high power for identifying individuals (PI = 8.4×10^{-18} including pure species of *A. mangium, A. auriculiformis* and their F_1 hybrid). In addition, the probability of identity between related individuals (PIsibs) which ranged between 2.6×10^{-5} to 5.7×10^{-7} for sets of pure species and the combination with their F_1 hybrids was also relatively small. PIsibs is the probability that two siblings from the same half-sib family will have the same genotype over all 16 loci. The PIsibs values in this study correspond to a 1 in 38,000 and 1.3 million chance that a seedling shared its genotype with another half-sib in a pure and mixed species population, respectively. Several studies have used marker sets with PIsibs probabilities las found here or even higher probabilities (Bellemain and Taberlet 2004; Costa et al. 2012; Hasenkamp et al. 2011). Thus, these sixteen markers should be suitable for a range of

applications in *A. mangium*, *A. auriculiformis* and their F_1 hybrids such as: fingerprinting clones, studying breeding systems, recovering male pedigrees from reproductively isolated seed orchards, checking the pedigree of controlled crosses.

2.5.2. Species differentiation

Bayesian modeling has become commonplace in studies assessing genetic structure within and between species (Anderson and Thompson 2002; Vaha and Primmer 2006) and to identify and quantify hybridisation between species (Adhikari et al. 2014; Barilani et al. 2007; Burgarella et al. 2009; Cullingham et al. 2013; David et al. 2002; Larcombe et al. 2014). Here we found that such a Bayesian modeling approach using the multiplexed SSR markers could be used to distinguish different pure and hybrid classes. Our simulation analysis showed that for pure species, pedigreed F_1 samples, and backcross samples, the accuracy and likelihood of classification was high. However, we had difficulty distinguishing F_2 samples from F_1 hybrids and backcross. This result is consistent with similar studies in forest trees (Burgarella et al. 2009; Larcombe et al. 2014) which also had most difficulty with differentiating F_2's from backcrosses.

The utility of the Bayesian approach was demonstrated in the discovery of five natural hybrid individuals (2.2%) in the "pure" species of CSIRO collections. These hybrids were found in provenances from Cape York Peninsula and PNG where the natural range of the two species overlaps. Three of these individuals were likely to be F_1's and two likely to be backcrosses. The leaf morphology of these individuals was checked following the molecular analysis, and it was found to be within the range of hybrid plants (Rufelds 1988), supporting their molecular classification. Hybrids between these species have been found previously in natural populations (Gunn et al. 1989a; Rufelds 1988) as well as in progenies from plantations (Kha 2001; Kha et al. 1997; Wickneswari and Norwati 1992). The two species

are synchronous in their flowering time and have the same suite of pollinator insects (Sedgley et al. 1992a). Thus, the likelihood of hybridisation is high when they are in proximity. Indeed, hybrids are common in production landscapes where both species are grown in plantations.

Despite the utility of the widely used Bayesian approach for hybrid identification, it can only ever produce probabilistic estimates of admixture based on essentially arbitrary cut-offs (*q-values*). In contrast, the six species diagnostic markers identified in this study provide an absolute measure of admixture. That is, these markers do not overlap in their alleles between our two focal taxa allowing easy discrimination of pure species, and hybrids. F_1 hybrids were shown to be heterozygous for all six loci, as expected. This diagnostic system could be used to provide a quick and powerful tool with direct applications in forestry. Since acacia hybrids have been widely planted with acacia hybrid breeding programs becoming more important (Harwood et al. 2015; Kha et al. 2012), these diagnostic markers enable accurate and quick identification of F_1 hybrids, allowing early disposal of non-F_1 individuals in their seedling stage thus delivering substantial saving in time and resources. These markers ensure that only true F_1 hybrid seedlings are used for field trials testing. Backcrosses and F_2 hybrids will also often be directly observable in individuals with mixed homozygous/heterozygous genotypes at these loci, however, discriminating between F_2 or backcross classes will remain difficult. At this stage, the breeding program for tropical acacias are focusing on pure species of *A.mangium* and *A. auriculiformis* and their F_1 clones (Harwood et al. 2015) so quality control could be carried out using these six diagnostic markers alone. Also distinguishing F_2's and backcrosses is not currently considered a primary concern, but if it does become necessary in the future, more loci could be added to increase discrimination power (Barilani et al. 2007; Vaha and Primmer 2006).

Appendix 2.1. List of wild populations of *A. mangium* and *A. auriculiformis* used in this study (seeds imported from ATSC).

Index	Seedlots	Locality	State	Latitude	Longitude	Alt. (m)
Acacia auriculiformis						
1	15477	MOREHEAD RIVER	QLD	15° 02'	143° 40'	70
2	18854	ARCHER R & TRIBS	QLD	13° 26'	142° 57'	90
3	19252	ROBERTS CREEK	QLD	15° 59'	145° 03'	200
4	15861	JARDINES GARDEN	QLD	10°47'	142° 29'	60
5	17705	OLIVE RIVER	QLD	12° 11'	142° 59'	4
6	16160	S ALIGATOR R	NT	13° 16'	132° 19'	40
7	16148	MANTON RIVER	NT	12° 50'	131° 07'	100
8	16149	DOUGLAS RIVER	NT	13° 51'	131° 09'	70
9	16158	GEROWIE CREEK	NT	13° 19'	132° 15'	100
10	18059/ 19390	POHATURI RIVER	PNG	09° 10'	142° 11'	40
11	18102/ 19262	MIBINI	PNG	08° 50'	141° 38'	18
12	17553/ 18963	BENSBACH	PNG	08° 53'	141° 17'	25

13	18058	SEWIRIMABU, FLY RIVER	PNG	08° 35'	143° 15'	20
14	18203	MAIKUSSA RIVER WP	PNG	08° 52'	142° 02'	10
15	19146	ARUFI	PNG	08° 43'	141° 55'	40
16	19391	BINATURI R WP	PNG	09° 00'	143° 00'	20
17	19392	BITURI R NW OF WIPIM	PNG	08° 37'	142° 42'	30
18	19684	PODARI VILLAGE WP	PNG	08° 52'	143° 53'	40
19	20136	MOREHEAD	PNG	08° 41'	141° 51'	15
20	20137	ORIOMO	PNG	08° 49'	143° 00'	10
A. mangium						
21	13231	NORTHWEST OF SILKWOOD	QLD	17° 42'	145° 57'	40
22	13239	SYNDICATE ROAD	QLD	17° 55'	145° 52'	50
23	13240	CARDWELL	QLD	18° 14'	145° 58'	60
24	13459	WEST OF MOREHEAD	QLD	08° 45'	141° 18'	30
25	13236	KURRIMINE	QLD	17° 46'	146° 05'	10
26	19232	WASUA	PNG	08° 19'	143° 02'	10

27	20131	LOWER FLY	PNG	08° 19'	143° 02'	10
28	15693	LANNERCOST SF INGHAM	QLD	18° 37'	145° 54'	170
29	17703	TULLY-MISSION BEACH	QLD	17° 55'	146° 05'	20
30	18083	AYTON-BLOOMFIELD	QLD	15° 53'	145° 20'	15
31	19214/ 13229	CLAUDIE RIVER	QLD	12° 44'	143° 16' /13'	30 / 60
32	19140	MALAM	PNG	08° 40'	142° 43'	45
33	18206	ARUFI VILLAGE WP	PNG	08° 43'	141° 55'	25
34	19139	BALIMO ARAMIA RIVER	PNG	08° 03'	142° 38'	15
35	19142	BINATURI	PNG	09° 02'	143° 04'	10
36	17868	MOREHEAD	PNG	08° 45'	141° 37'	40
37	15367	7K SSE OF MOSSMAN	QLD	16° 31'	145° 24'	60
38	18212	BENSBACH AREA WP (PNG)	QLD	08° 53'	141° 17'	25
39	18214	KAPAL VILLAGE / ORIOMO	PNG	08° 37'	142° 47'	40
40	19611	MAKAPA	PNG	07° 56'	142° 35'	15
41	19730	LAKE MURRAY WP	PNG	07° 00'	141° 33'	50

42	19733	BITURI PROV WP	PNG	08° 40'	142° 43'	45
43	19736	UPPER ARAMIA PROV WP	PNG	07° 56'	142° 35'	15
44	20132	WIPIM – ORIOMO	PNG	08° 49'	143° 00'	10
45	20133	PONGAKI - ORIOMO REGION	PNG	08° 40'	142° 43'	45
46	19297	REX-CASSOWARY	QLD	16° 31'	145° 24'	60

NT: Northern Territory, Australia; NSW: New South Wales, Australia; PNG: Papua New Guinea; QLD: Queensland, Australia

Appendix 2.2. List of control crossed samples used for validating markers (2 samples/combination) (Am= *A. mangium*, Aa = *A. auriculiformis*)

Types of cross		Types of cross	
I	**Aa x Am**	**II**	**Am x Aa**
1	6 x 82	10	30 x 156
2	6 x 63	11	30 x 84
3	85 x 14	12	35 x 6
4	85 x 30	13	63 x 6
5	156x 82	14	63 x 156
6	156 x 113	15	63 x 85
III	**Aa x Aa**	16	82 x 6
7	6 x 85	17	82 x 156
IV	**Am x Am**	18	82 x 84
8	63 x 30	19	82 x 85
V	**Aa X Am**	**VI**	**Aa X Am**
9	3 x 20	20	6 x 22

Appendix 2.3. Primer sequences of 40 published microsatellite loci

Index	Primer Name	Forward Primer	Reverse Primer	Source
1	AH01	TTGAGGTTGAGGGTGATGAA	GGCAAGCCTCTCTCTCTCT	(Ng et al. 2005)
2	AH02	TGAACGGCTCTCTCTCTCT	TTCATCACCCTCAACCTCAA	
3	AH08	TTCAGGCCTCTCTCTCTCT	TCGCCTAAATCCTTCCCAAC	
4	AH16	GAGGGTAATGCTTCAAGTAGAC	TGCGTGTCTCCCCACTACTC	
5	AH18	GGCGCAACTCTCTCTCTCT	TTGGTCACTTAGCGCATGCC	
6	AH20	GGCGAGACTCTCTCTCTCT	CCTGTCAACCCTGAATCATT	
7	AH28	GTGAAGGCTCTCTCTCTCT	GGAGATGGATAGAGATGGCC	
8	AH29	GGCCATCTCTATCCATCTCC	CCTTCCCCAATTCCTTTGCTTC	
9	AH37	GTCGCGTACACAGACACAGT	GGCGCACCTCTCTCTCTCT	
10	AH54	AATGAACAAGAGCCATCACAG	GGTATGGTGTTTCTATGAGCTATC	
11	AH56	GATAGCTCATAGAAACACCATACC	GGCGAAGCTCTCTCTCTCTCTCTCTCT	
12	AH59	CTACGAGGCACAAAAGTT	GTGAGGGCTCTCTCTCTCTCTCTCTCT	
13	AH69	CTCTACCAGCTTCATTCGTC	TCCACTCTTCCCTCTCAACT	
14	AH71	GGGGGAGCTCTCTCTCTCTCTCTCTCT	GCTACTAAGGTTTCTTTCACGG	
15	AH76	GGGGAGGCTCTCTCTCTCTCTCTCTCT	GTGACCTGAGTTAGGAAGGAGC	
16	AH2_1	GACAGAGGGAGCATTTTGTA	CAGACAAGACCAGAGAATGAC	(Aggarwal et al. 2011)
17	AH2_2	CGGTTTAGCAGTCACAGAAG	TACAAGCATCATCATGGAAG	
18	AH2_5	TGAGTCAACCAACTTCCTTC	CGATTCGCAACTAAAAGTG	
19	AH2_6	ACAGTGGTATGATGGGAGTG	CACTAACGTCACAATGATCG	
20	AH2_11	CAAGCGTTCAGTAGAGGTTT	CCCTGTAGATATAAGCACTGAAC	
21	AH2_12	CTTGTATTTCCATGGTGAGTC	AGCGATTGATATCCTTGAGA	

22	AH2_13	GAAGAAGCAGGAGGAGGTAG	TGTTTTCCACTTCTCACACA	
23	AH2_17	AAATTCTCTTCGCAACCAC	TCTGAGGTATTCCATTAGGC	
23	AH3_1	CTAAGGCACTTGGATCATTC	AGAGAGAGAGAGGCACACTG	
25	AH3_4	GATCTCAGCAGCAGCAAC	CTGGTGGATGTGGTTTGG	
26	AH3_6	AGCCAAGGTTGAGACTGTAA	TCCTTTTTCTCAGCTTTGTC	
27	AH3_10	AGGGATATCGGATGCTTACT	AAAGATGCAGCAGACCTATC	
28	AH3_13	GAGGGATGAGATCTGTTTGA	CACCGAAATCATCAGGATAG	
29	AH3_17	AATACTGGCATTTCGTGTCT	AACAAAACATCACCAAGGTC	
30	AH2_4	GGATTATAAATGGCTGATCG	TGGTCCCCTAACTACAAATG	
31	AH2_9	CGTCTCATCGATCTTCTTTC	GGAGGCATAACATCAAACAT	
32	AH2_10	CAGTGTTGTGGTCCTTTTTC	TTTACTCTCGAGCAAACACC	
33	AH2_14	CGGAAGAAGAAGAAGAAGAA	AATACAGCACTTGGCAACA	
34	AH2_15	TCCGAAATGTTGAACTAAGG	TATGAAAGCCAACCAGAAAC	
35	AH3_18	TGAGACAATTAATGGTGGTG	TTTACAAGGGAAAAGCTGAG	
36	Am041	TAGGCTAATGGTCATATTCCTAG	AGAGATAGGGGTACACACTAAAAAAC	(Butcher et al.
37	Am387	TGATACAAGGGAAGACAGAGTGG	CCAACTCAAAACCTGACAACG	2000a)
38	Am456	TGGGTATCACTTCCACCATT	AGGCTGCTTCTTTGTGCAGG	
39	Am014	GATCTAACGTTGCTATATGAGGAAAGG	CTGGTTGTTCGCTTATATGG	
40	Am164	ACCCGGACGTATAGAAATAAATACA	CGTGGAGGCAAGCAATATC	

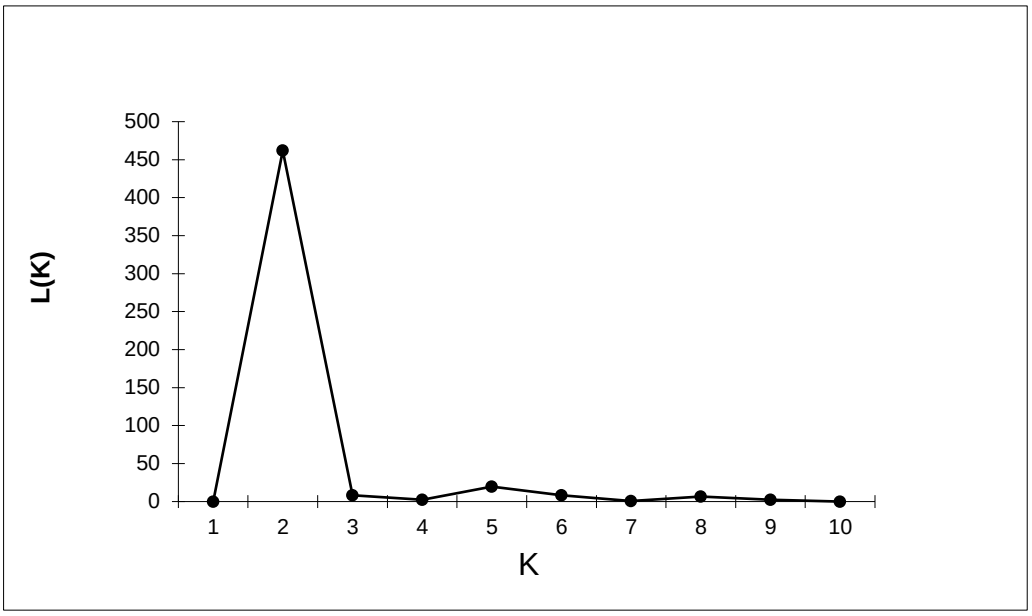
Appendix 2.4. Mixes of 16 markers used in second stage of genotyping

Index	Marker	Repeat motif	Forward Primer	Reverse Primer	Ta[1]	Allele size (bp)	Dye
1	PCT4 & ancp8a	$(CT)_7$	KKVRVRV(CT)6	TCGCCTAAATCCTTCCCAAC		93-97	6-FAM
2	ancp16a & ancp17s	$(GA)_{16}$	TGCGTGTCTCCCCACTACTC	GAGGGTAATGCTTCAAGTAGAC		97-115	HEX
3	ancp69a & ancp70s	$(GA)_8$	TCCACTCTTCCCTCTCAACT	CTCTACCAGCTTCATTCGTC	50	91-99	NED
4	AH16	$(GA)_{16}$	GAGGGTAATGCTTCAAGTAGAC	TGCGTGTCTCCCCACTACTC		98-110	ROX
5	AH3_6	$(AAG)_5$	AGCCAAGGTTGAGACTGTAA	TCCTTTTTCTCAGCTTTGTC		235-243	6-FAM
6	AH08	$(CTs_7$	TTCAGGCCTCTCTCTCTCT	TCGCCTAAATCCTTCCCAAC		93-97	6-FAM
7	AH69	$(GA)_8$	CTCTACCAGCTTCATTCGTC	TCCACTCTTCCCTCTCAACT	52	91-95	HEX
8	AH29	$(GA)_{10}$	GGCCATCTCTATCCATCTCC	CCTTCCCCAATTCCTTTGCTTC		109-117	6-FAM
9	ancp29s & ancp30a	$(AG)_{10}$	GGCCATCTCTATCCATCTCC	CCTTCCCCAATTCCTTTGCTTC		119-125	ROX
10	ancp54s & ancp55a	$(AG)_7$	AATGAACAAGAGCCATCACAG	GGTATGGTGTTTCTATGAGCTATC		58-70	NED
11	AH54	$(GA)_6$	AATGAACAAGAGCCATCACAG	GGTATGGTGTTTCTATGAGCTATC	56	62-76	HEX
12	AH3_17	$(TTC)_7$	AATACTGGCATTTCGTGTCT	AACAAAACATCACCAAGGTC		208-214	ROX

13	AH76	$(CT)_{10}T(CT)_4$	GGGGAGGCTCTCTCTCTCTCTCTCTCT	GTGACCTGAGTTAGGAAGGAGC		130-146	NED
14	Am 465		TGGGTATCACTTCCACCATT	AGGCTGCTTCTTTGTGCAGG	60	142-158	6-FAM
15	Am 387		TGATACAAGGGAAGACAGAGTGG	CCAACTCAAAACCTGACAACG		188-202	NED
16	Am 041		TAGGCTAATGGTCATATTCCTAG	AGAGATAGGGGTACACACTAAAAAAC		145-149	HEX

(^1T_a: annealing temperature (^0C))

Appendix 2.5. Summary of simulations for K=1 to 10 from STRUCTURE for Evanno approach for wild population of *A. auriculiformis*, *A. mangium* and their putative hybrids.



Chapter 3 Using SSR markers for hybrid identification and resource management in Vietnamese Acacia breeding programs

3.1. Abstract

We used a set of 16 SSR markers to check the identity of pure-species and hybrid clones in Vietnam's *Acacia auriculiformis*, *A. mangium* and acacia hybrid (*A. mangium* x *A. auriculiformis*) breeding programs. The HIest statistics package (Gross and Schiestl 2015), applied to a large synthesised population, enabled accurate allocation of genotypes to the two pure species, F_1 and F_2 interspecific hybrids and backcrosses, based on estimates of hybridity and heterozygosity. The hybridity status of putatively pure *A. mangium* and *A. auriculiformis* clones in adjacent clonal seed orchards was checked. Four out of 100 clones selected as *A. mangium* were found to be backcrosses (*A. mangium* x F_1 interspecific hybrid) while out of 96 clones selected as *A. auriculiformis* two were F_1 hybrids and two were backcrosses (*A. auriculiformis* x F_1 hybrid). The markers were then applied to check the hybridity status of 160 putative acacia F_1 hybrid genotypes that had been selected on morphological criteria from open-pollinated progenies collected from *A. auriculiformis* and *A. mangium* parents. Many selections based on morphology were found to be mistaken. Only thirteen of 63 clones originating from *A. auriculiformis* mothers were F_1 hybrids, four were backcrosses and the remaining 46 were pure *A. auriculiformis*. Fewer mistakes were evident for clones selected from *A. mangium* mothers, with 82 out of 89 clones confirmed as F_1 hybrids, three as backcrosses and four as pure *A. mangium*. The occurrence of F_1 hybrids and backcrosses in pure species seed orchards and their progeny shows that inter-species contamination is an issue requiring management in both pure-species and hybrid breeding of these species in Vietnam. Examination of genetic distances among verified

clones showed patterns of relatedness that were consistent with pedigree records. Implications for resource management as well as for breeding and clonal selection strategies are considered.

3.2. Introduction

Acacia mangium, A. auriculiformis and *A. crassicarpa* are tropical acacia species which occur naturally in Northern Australia and New Guinea and have displayed outstanding adaptability and productivity in plantations in the humid tropics. By 2013, Vietnam had established about 1.1 million ha of acacia plantations, including 600,000 ha of *A. mangium*, about 400,000 ha of clonal plantations of the natural F_1 hybrid between *Acacia mangium* and *A. auriculiformis* (hereafter referred to as acacia hybrid), 90,000 ha of *A. auriculiformis* and about 5,000 ha of *A. crassicarpa* (Griffin et al. 2011; Nambiar and Harwood 2014). Acacia plantations are managed to produce pulpwood and small sawlogs on rotations of 5 - 10 years (Harwood et al. 2015).

Acacia breeding in Vietnam commenced in the mid-1990s with the establishment of first generation progeny trials of *A. auriculiformis* and *A. mangium* (Kha 2001). Some progeny trials were converted to SSOs by selective thinning. Open-pollinated seeds were then collected from the best trees of superior families to establish second-generation progeny tests (Harwood et al. 2015). One hundred and fifty *A. mangium* and 120 *A. auriculiformis* trees were selected at age three in progeny trials planted in southern Vietnam in 1997. These selections were cloned and planted in adjacent CSOs in 2001, to produce improved pure-species and hybrid seed (*A. mangium* x *A. auriculiformis*) (Hai et al. 2008) (Fig. 3.1).

Acacia hybrid (*A. mangium* x *A. auriculiformis*) is important for wood production because of its high growth rate, acceptable wood properties and wide adaptability to various environments (Kha 2001). Natural hybrids between these two parent species are frequently

produced when they grow in adjacent stands (Kha 2001). No other tropical acacia species present in Vietnam is known to hybridise with these taxa (Harwood et al. 2015). Consequently, only these two species contribute to the occurrence of acacia hybrids in Vietnam, in contrast to the situation with eucalypts where many species hybridise in exotic plantings (Dehon et al. 2013). Commencing in 1992 in Vietnam, selection and testing aimed to identify fast growing hybrid clones (Kha et al. 2012). In addition to fast growth, good stem straightness and light branching, wind tolerance and disease resistance have been used as selection criteria for acacia hybrid clones (Harwood et al. 2015). However, only about ten acacia hybrid clones are currently planted on a significant scale in Vietnam (ND Kien, pers. comm).

Using a small number of highly productive clones in forestry has the potential for high yield and simple management. However, there is risk that some or all of these clones may be susceptible to biotic hazards or climate extremes (Burdon and Aimers-Halliday 2006; Roberds and Bishir 1997). Until recently, disease and pest attack has had little impact on acacia plantations in Vietnam, however, this may change in the near future. *Ceratocystis*, a fungal wilt disease that kills acacia trees by infecting stem tissues and disrupting flow of water to the tree crown (Tarigan et al. 2011), has caused damage and death in acacia plantations in several different locations throughout South East Asia including Vietnam (Thu et al. 2014).

Acacia hybrid progenies can be produced via control pollination (Nghiem et al. 2016). However, acacia flowers are very small, difficult to emasculate (Griffin et al. 2010) and the seed yields from crossing are low, usually less than 10 seeds per 100 pollinated flowers (Nghiem et al. 2016). Crowns of selected parents must also be accessed using scaffolds or following their capture into clone banks, so it is difficult to produce more than a few crosses per year. Therefore, production of new acacia hybrid genotypes for testing has mainly relied

on detection of hybrid individuals within open-pollinated progenies sourced from adjacent stands of the two species (Harwood et al. 2015).

An acacia hybrid selection program managed by the Vietnamese Academy of Forest Sciences (VAFS) is now testing over 500 hybrid candidates selected from open-pollinated progeny raised from open-pollinated seed collected from unpedigreed seed production areas (SPAs), CSOs and SSOs. Guidelines for nursery selection of hybrids were first developed by Rufelds (1988) and improved by Gan and Liang (1992). Leaf taxonomic characteristics and leaf development pattern as well as number of pinnate leaves of young seedlings at 7 to 10 weeks old were used to detect hybrid candidates. When seedlings were raised from mother trees of *A. mangium* and *A. auriculiformis* growing in adjacent stands in Malaysia, 46.6% of *A. mangium* and 39.0% of *A. auriculiformis* progeny were identified as hybrids (Gan and Liang 1992). However, identifying candidate hybrids at the nursery stage based on seedling morphology is challenging, especially when screening progenies from *A. auriculiformis* mothers due to the high variation of leaf morphology of this species (Pinyopusarerk et al. 1991; Shukor et al. 1994; Widyatmoko and Shiraishi 2003). Morphology assessment in four nurseries over-estimated the frequency of hybrid individuals in progenies of both parent species by more than 10%, compared with the proportions of hybrids determined in samples of the same seedlots using the isozyme marker *Gdh-1* (Gan and Liang 1992). In Vietnam, the detection of hybrids is now complicated by pollen dispersal from acacia hybrid plantations that are frequently planted in close proximity to pure-species SPAs, SSOs and CSOs (Harwood et al. 2015) potentially yielding a complex mix of advanced generation hybrids (Le et al. 2016).

Molecular markers offer the prospect of more accurate hybrid identification and have been used for F_1 acacia hybrid identification, for example, the isozyme marker *Gdh-1* (Wickneswari 1989), RAPD markers (Wang and Hu 1996), two SCAR markers (Huang et

al. 2005) and five SNP markers (Yuskianti et al. 2011a). However, there are no published reports of these markers being applied in breeding programs.

Recently, a set of 16 SSR markers, including ten highly polymorphic SSR and six species-diagnostic markers, has been developed in order to distinguish *A. mangium*, *A. auriculiformis* and their interspecific hybrids. This SSR set has been used to genotype range-wide samples of *A. mangium, A. auriculiformis* and their F_1 hybrids. It has also been used to identify F_1 hybrid and pure individuals of these two species in admixed populations (Le et al. 2016).

The HIest statistical package was designed for hybrid discrimination (Fitzpatrick 2012), in situations where F_2's co-occur with F_1's and backcross generations. This package has proved its utility in hybrid zone research, having been used to estimate the ancestry of admixture in salmon (Glover et al. 2013b), whales (Glover et al. 2013a) and several plant species (Arntzen et al. 2014; Kawakami et al. 2014; Michalcová et al. 2014). This package along with a set of informative SSR markers offers the potential for identification and characterisation of acacia hybrids from open-pollinated seed sources. Here, we report the use of this set of SSR markers together with the HIest package to check the genetic status of pure species and hybrid clones used in Vietnam's tropical acacia breeding programs. The utility of the SSR markers in studies of clonal identity and genetic diversity is also investigated. The implications of the results for resource management and ongoing pure-species and hybrid breeding are discussed.

3.3. Materials and methods

3.3.1. Plant materials

In this study we first checked the genetic status of 100 *A. mangium* and 96 *A. auriculiformis* clones in the adjacent CSOs planted in 2001 at Bau Bang in Binh Duong Province, southern Vietnam. These two CSOs functioned initially as clone trials (Hai et al. 2008). They were selectively thinned in 2004 to retain the best-performing 100 *A. mangium* and 96 *A. auriculiformis* clones, after which seed collections yielded some of the hybrid candidate genotypes that we analysed (Fig. 3.1 and Table 3.1).

Secondly, we investigated the hybridity status of 160 randomly chosen clones from among the recently selected 500 putative hybrid genotypes currently under clonal testing in field trials. These candidates had been selected based on intermediacy in leaf and phyllode morphology compared to their parent species at either of two ages: (1) at three months in the nursery in open-pollinated progenies from pure-species *A. mangium* and *A. auriculiformis* seed orchards, followed by re-selection at two years in field trials of the selected seedlings (two-stage process); or (2) at two years in progeny trials of the two species (one-stage process) (Harwood et al. 2015) (Fig 3.1). The traits used for hybrid selection at the nursery stage were aspects of the guide developed by Gan and Liang (1992). Sixty-three of these candidates were from *A. auriculiformis* mothers, 89 from *A. mangium* mothers and 8 of uncertain maternal origin (Table 3.5). Twenty-four of the hybrid candidates originated from known and genotyped clones in the 2001 *A. mangium* and *A. auriculiformis* CSOs described above. To check for errors in propagation and clone bank management we also genotyped a second ramet in the clone bank for each of these 24 candidate clones. We also checked the hybridity status of ten commercially planted hybrid clones, eight of which were descended from *A. mangium* and two from *A. auriculiformis* mothers. These ten clones are widely planted throughout Vietnam (Table 3.5 and Fig. 3.1).

Table 3.1. Plant materials and the origins of the clones

Species	Types	Planted as	Total	Origin of the clone			
				Natural provenances	SPA	SSO	CSO
A. mangium	Clone	CSO	100	65	0	35	0
A. auriculiformis	Clone	CSO	96	31	0	65	0
Candidate hybrid	Clone	Clonal trials	160	0	19	117	24
Acacia hybrid	Clone	plantations	10	10	0	0	0

SPA: Seed Production Areas, SSO: Seedling Seed Orchard, CSO: Clonal Seed Orchard

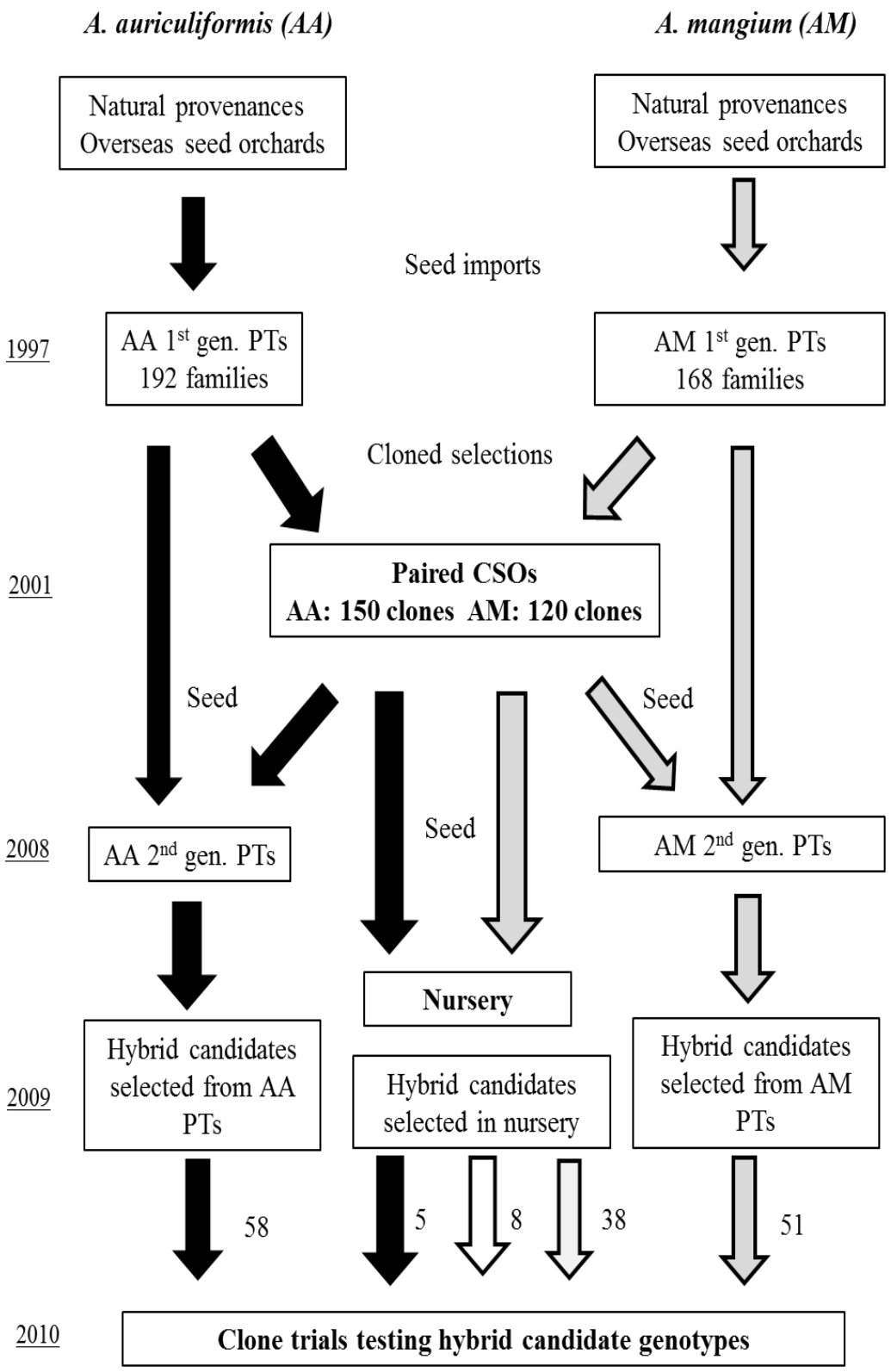


Figure 3.1. Acacia breeding programs in Vietnam showing the origins of samples used in this study (CSO: clonal seed orchard, PT: progeny trial). Eight of the candidate hybrid clones selected in the nursery had unknown female parentage.

3.3.2. DNA isolation, PCR conditions and PCR product analysis

Phyllode samples were collected from clone banks of the pure-species and hybrid clones at the VAFS field station at Bavi (Hanoi), which had been used to provide the clonal ramets used in the field trials (Table 3.1 and Fig. 3.1). Phyllodes were dried with silica gel (or overnight in an oven at 50^0C) and stored at room temperature before DNA isolation. DNA was extracted using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), where 50 mg of dried phyllode tissue was crushed to a powder using mortar and pestle and liquid nitrogen. The standard Qiagen protocol was used (www.qiagen.com/handbooks). DNA concentration and purity were assessed using gel electrophoresis and comparison with a Lambda *Hin*dIII molecular weight standard.

PCRs were performed in a GeneAmp PCR system 9700 (Applied Biosystems, USA) with a final volume of 12.5 µl, consisting of 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primer, 0.5 U Taq DNA polymerase (Invitrogen, Massachusetts, USA) and 20 ng of genomic DNA. Thermocycler conditions and annealing temperature ranged from 50^0C to 60^0C depending on each marker (Le et al. 2016). The 16 SSRs were tagged with fluorescent dyes NED, 6-FAM, HEX and ROX on their forward primers and PCRs were conducted in four multiplex mixes (Le et al. 2016). PCR products were separated using an ABI 3730 DNA Analyser (Applied Biosystems, USA) by the company 1st BASE (www.base-asia.com); raw data were analysed using GeneMapper 3.7 (ABI, USA) software to score genotypes.

3.3.3. Data analysis

In order to test the power of the 16 SSR markers in detection of hybridity status of individuals in an admixed population, we used the R package HIest (Fitzpatrick 2012) to calculate maximum likelihood estimates of ancestry (S, "the axis that arranges all hybrids between

two ancestral extremes") and inter-species heterozygosity (HI, "the axis that distinguishes F_1, F_2, backcrosses and recombinant inbred lines"). We used the allele frequencies from the 127 pure-species *A. mangium* and 96 pure-species *A. auriculiformis* samples analysed by Le et al. (2016), which were open-pollinated progenies collected by the Australian Tree Seed Centre from native provenances, and the program HYBRIDLAB (Nielsen et al. 2006) to simulate 1,000 individuals for each of the following populations: pure *A. mangium* (AM), pure *A. auriculiformis* (AA), F_1, outcrossed F_2, the first backcross of F_1 with *A. mangium* (BCM) and that with *A. auriculiformis* (BCA). These 6,000 simulated genotypes were then run in the HIest package with the following parameters: 1,000 iterations per population and a startgrid of 20, to calculate maximum likelihood estimates of ancestry (S) and inter-species heterozygosity (HI) for each genotype.

Genotypes (either simulated or real) were identified as pure *A. mangium* if S = 0 and HI = 0, and classed as pure *A. auriculiformis* if S = 1 and HI= 0. Genotypes were classed as F_1 if S = 0.5 and HI = 1, and as F_2 if $0.25 < S < 0.75$ and HI near 0.5. A genotype was identified as a backcross if it was distributed on the side of the triangle (see Fig. 3.2) with HI near 0.5 and $S < 0.5$ for backcross with *A. auriculiformis* (average near 0.25) and $S > 0.5$ for backcross with *A. mangium* (average near 0.75) (Fitzpatrick 2012).

Additionally, STRUCTURE (Pritchard et al. 2000) was used for hybrid detection in the simulated populations in order to compare results with those obtained using HIest. STRUCTURE was conducted as in a previous study (Le et al. 2016), using an *a priori* hypothesis that K=2 (two species) with a burn-in of 100,000 Markov Chain Monte Carlo (MCMC) iterations with subsequent 100,000 data generating iterations. To identify the F_1 hybrids using STRUCTURE, we used a q_{value} (genotype membership) cut-off of $q_{value} = 0.5 \pm 0.10$. First generation backcross were those with $q_{value} = 0.75 \pm 0.15$, and if a sample had

$q_{value} > 0.9$ in one cluster, the individual was classified as a pure species genotype (Le et al. 2016; Pritchard et al. 2000).

The HIest package (Gross and Schiestl 2015) was also used to verify the genetic status of 100 *A. mangium* and 96 *A. auriculiformis* clones in the CSOs as well as classifying the hybridity degree of 160 hybrid candidate clones.

A chi-square test for independence was used to determine whether there was a significant difference between *A. mangium* and *A. auriculiformis* in the frequency of mistakes in the morphology-based assessment used to select the candidate clones. The null hypothesis was that accuracy of selection was the same for both species.

We also undertook analysis of genetic relationship between siblings derived from the same mother tree in comparison with unrelated genotypes. In order to analyse the genetic distances between families and between sibling clones within open-pollinated families. Genetic distances (Nei 1972) between and within families were calculated for 36 *A. auriculiformis* and 21 *A. mangium* families which had contributed two or more clones to the pure-species CSOs using the GelAlex V6.5 program (Peakall and Smouse 2012).

The genetic diversity in the set of newly selected acacia hybrid clones determined to be 'confirmed' F_1's in this study was compared to that of the ten commercial hybrid clones. The average number of alleles per locus and private alleles as well as allelic richness (standardised based on a minimum sample size of ten), were calculated with FSTAT V2.93 (Goudet 1995). Observed heterozygosity and expected heterozygosity were determined by GDA V1.2 (Lewis and Zaykin 2001).

In order to analyse the genetic structure within the population of F_1's we undertook analyses of the genetic distance between clones. The genotypic distances between the confirmed newly selected F_1's and the 10 commercial hybrid clones were estimated by GelAlex V 6.501. Principal Coordinates Analysis (PCoA) was undertaken with GelAlex V 6.501 via distance

matrix with data standardisation (Peakall and Smouse 2012). In addition, Principal Coordinates Analysis (PCoA) and genotypic distance within and between full-sib families in the set of 50 progeny derived from 20 controlled crosses between seven *A. auriculiformis* and nine *A. mangium* parents (Le et al. 2016), was also estimated by GelAlex V6.501, in order to estimate the genetic distance at family levels. Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was used to partition the genetic variation between two new hybrid groups (new clones from *A. mangium* mothers, new clones from *A. auriculiformis* mothers) and among individuals within each group.

To evaluate the power of the set of 16 SSR marker in clonal identification, Probability of Identity (PI) were estimated by GelAlex V 6.501 (Peakall and Smouse 2012) for the set of 160 hybrid clones.

3.4. Results

3.4.1. Power of the markers and HIest package in discriminating hybridity status

The HIest analysis of the simulated generations (including pure species, F_1, F_2 and backcross) showed that the 16 SSR markers had good power in discriminating the hybridity status of *Acacia* admixture (Table 3.2 and Fig. 3.2). All simulated pure *A. mangium* and *A. auriculiformis* and all F_1's were classified with an accuracy of 100%. Most of the simulated F_2 individuals (92%) were classified correctly and most of the remaining were misclassified as backcrosses. In addition, 92 - 93% of the simulated backcross individuals were identified as backcross genotypes and most of the remaining samples were misclassified as F_2's. Using STRUCTURE, pure-species, F_1 and backcross individuals were again correctly assigned (100% accuracy) but discrimination between F1 and F_2 was impossible because they have the same q-value cut-off. Furthermore, the accuracy of identification of backcrosses was lower than using HIest (Table 3.2).

Table 3.2. The accuracy of assigning simulated individuals to the correct hybrid or pure species generation based on their predicted ancestry (S) and heterozygosity (HI) using the HIest package compared to classification using STRUCTURE

Simulated generation	Parameters	AM	AA	F1	F2		BCM	BCA	Accuracy (%)
HIest	Predicted S	Predicted HI							
AM	0.0 – 0.1	0	100	0	0	0	0	0	100
AA	0.9 – 1.0	0	0	100	0	0	0	0	100
F_1	0.5	1.0	0	0	100	0	0	0	100
F_2	0.25-0.75	Near 0.5	0	0	1	92	3	4	92
BCM	Near 0.25*	Near 0.5	0	0	0	7	93	0	93
BCA	Near 0.75*	Near 0.5	0	0	2	6	0	92	92
STRUCTURE	$q_{cut\text{-}off}$								
AM	0.0 – 0.1	100	0	0		0	0	0	100
AA	0.9 – 1.0	0	100	0		0	0	0	100
F_1	0.5± 0.10	0	0	100		0	0	0	100
F_2	0.5± 0.15	0	0	-		-	10	8	-
BCM	0.25± 0.15	1	1	2		10	86	0	86
BCA	0.75± 0.15	0	0	5		8	0	87	87

(* see Fig. 3.2, AM: *A. mangium*, AA: *A. auriculiformis*, BCM: backcross with *A. mangium*, BCA: backcross with *A. auriculiformis*)

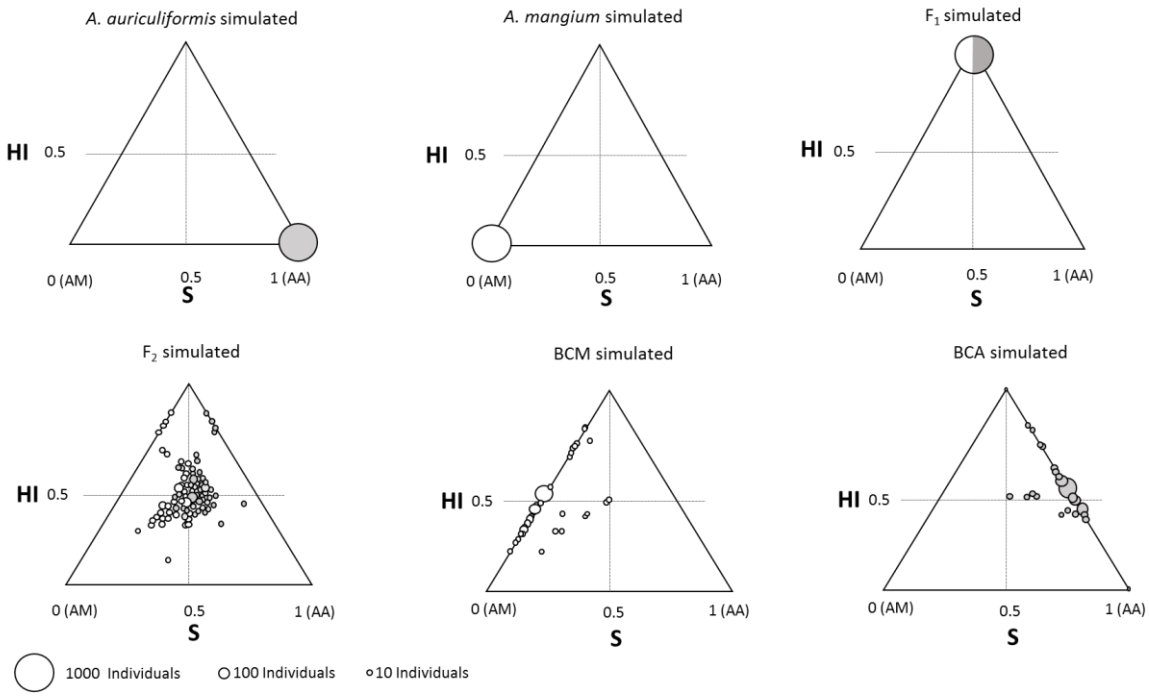


Figure 3.2. Estimates of ancestry (S) and heterozygosity (HI) using HIest in simulated populations of acacias (AM: *A. mangium*, AA: *A. auriculiformis*, $F_{1:}$ first generation hybrid between AA and AM, $F_{2:}$ second-generation hybrid, BCM: backcross between F_1 and *A. mangium*, BCA: backcross between F_1 and *A. auriculiformis*)

3.4.2. Purity status and genetic structure in the CSOs of A. auriculiformis and A. mangium

Overall, 4% of the putatively pure-species *Acacia* clones were found not to be pure. Two of the 96 putative *A. auriculiformis* clones were identified as F_1 hybrids, two were classified as backcrosses between F_1 hybrids and *A. auriculiformis*, and 92 were confirmed as pure-species *A. auriculiformis* genotypes (Table 3.3 and Fig. 3.3). Four out of the 100 putative *A. mangium* clones were classified as backcrosses between F_1 hybrids and *A. mangium*, while 96 were confirmed as pure-species *A. mangium*.

Table 3.3. The status of clones from the clonal seed orchards of *A. mangium* and *A. auriculiformis* based on HIest analysis

	Total number of clones	Genotypes		
		Pure species	Backcross	F_1
A. auriculiformis	96	92	2 (BCA)	2
A. mangium	100	96	4 (BCM)	0

The *A. auriculiformis* population in the CSO appears to have captured more genetic variation than that of *A. mangium.* The average genetic distance (Nei 1972) between families represented in the CSOs was slightly higher in *A. auriculiformis* (0.14), than in *A. mangium* (0.11). Genetic distances among clones within families were much lower in both *A. auriculiformis* (mean 0.02) and *A. mangium* (mean 0.01) (Table 3.4). Within the 50 control pollinated hybrid progeny, genetic distances between families were consistently greater (mean 0.13) than among full-sibs within families (mean 0.008). As expected, the differences within control pollinated families were less than those within the open-pollinated families of *A. mangium* and *A. auriculiformis* (Table 3.4).

Table 3.4. Genetic distance within and between family members in CSOs and control pollinated F_1 hybrids (CP F_1)

	Number of families with two or more sibs	Genetic distance between sibs within family			Genetic distance between families		
		Min	Mean	Max	Min	Mean	Max
A. auriculiformis	37	0.01	0.02	0.03	0.09	0.14	0.21
A. mangium	25	0.01	0.01	0.02	0.05	0.11	0.18
CP F_1	20	0.01	0.01	0.01	0.07	0.13	0.16

3.4.4. Status of candidate hybrid clones

Many of the candidate hybrid clones were found to have been misclassified. From the set of 160 candidates genotyped, 95 were found to be F_1 acacia hybrids. Out of the 63 candidates from *A. auriculiformis* mothers, 46 were classified by HIest as pure species, four were classified as BCA and only 13 were classified as F_1 acacia hybrids. However, mis-classification was less common when *A. mangium* was the mother; 83 out of 89 candidates were confirmed as F_1 acacia hybrids, four were classified as pure *A. mangium* and three were classified as BCM (Table 3.5 and Fig. 3.3). The rates of misclassification differed significantly between the two maternal origins (*A. mangium* and *A. auriculiformis*) (d.f. = 1, P = 0.0005). Eight acacia hybrid candidates of unknown maternal origin were also genotyped, four were confirmed as F_1 acacia hybrid, three as pure *A. auriculiformis* and one as BCM. As expected all ten commercial acacia hybrid clones were classified as F_1 hybrids (Table 3.5).

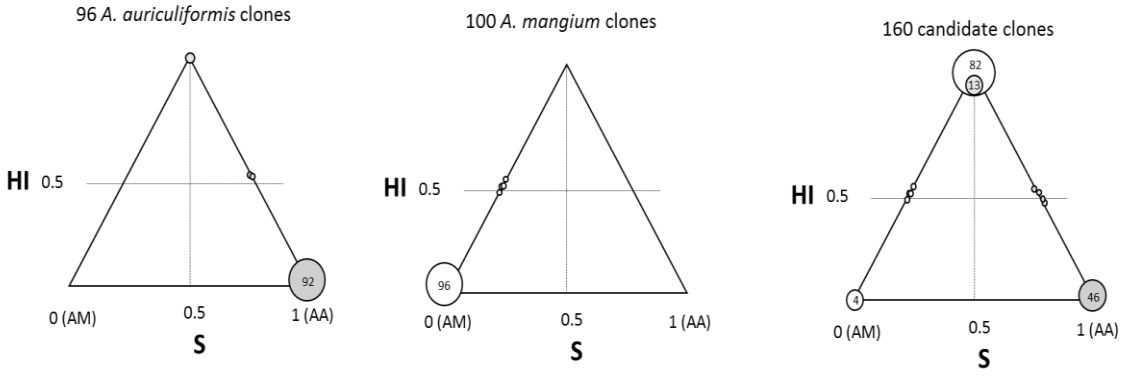


Figure 3.3. Estimates of ancestry (S) and heterozygosity (HI) of 100 *A. mangium* clones, 96 *A. auriculiformis* clones in the clonal seed orchards (CSO) and 160 candidate hybrid clones using HIest

Table 3.5. Genotypes of 160 F_1 acacia hybrid candidates and 10 commercial clones assessed using a set of 16 SSR markers and HIest package

Maternal parent	Genotypes of candidate clones					
	Total	AA	BCA	F_1	BCM	AM
A. auriculiformis	63	46	4	13	0	0
A. mangium	89	0	0	82	3	4
Unknown mother	8	3	0	4	1	0
Commercial hybrid clones	10	0	0	10	0	0
Overall total	170	49	4	109	4	4

3.4.5. Clonal and maternal checking

Among the 160 candidate acacia hybrid selections two were found that had the same genotype in all 16 markers and were considered as one hybrid (PI= 1.2×10^{-6}). This matched the original selection records (subsequently checked), which confirmed that a mistake had been made in setting up two separately identified clones from a single tree. For each of the 48 candidates from which two ramets were sampled in the clone bank, the duplicate samples had identical genotypes.

Each of the 24 clones descended from known *A. mangium* and *A. auriculiformis* mothers that were genotyped in this study had a genotype consistent with that of their mother, with at least one allele per locus matching an allele from their mother at all 16 marker loci (data not shown).

3.4.6. Genetic diversity of new hybrid clones

There was no difference in genetic diversity between the subset of new hybrid clones selected from *A. mangium* mothers and those derived from *A. auriculiformis* mothers (Table 3.6). H_e and H_o were identical and allelic richness was not significantly different ($P > 0.05$). However, the genetic diversity of the new hybrid clones was higher than that of the commercial clones. The set of 95 confirmed F_1 hybrid clones displayed higher levels of genetic diversity than the set of 10 commercial clones when measured by average number of alleles per locus, average number of private alleles and allele richness ($P < 0.01$) (Table 3.6, Fig. 3.4). There were no significant differences in H_e and H_o between the new hybrid clones and commercial clones ($P > 0.05$). AMOVA also indicated that the difference between the new and commercial subsets of hybrids accounted for only 9% of the variation ($P < 0.01$) (data not shown) and that most of the genetic variance (91%, $P < 0.01$) was within subsets.

Table 3.6. Comparison of genetic diversity indices between new and commercial acacia hybrid clones

Group	N_i	N_a	N_p	R_t	H_e	H_o
New hybrid clones from AM	82	9.1	5.6	6.1	0.74	0.96
New hybrid clones from AA	13	6.3	4.9	5.9	0.74	0.96
Commercial hybrid clones	10	4.9	1.0	4.9	0.68	0.93

(N_i: number of sample, N_a: average number of alleles per locus, N_p: average number of private alleles, R_t: allelic richness, H_e: expected heterozygosity, H_o: observed heterozygosity)

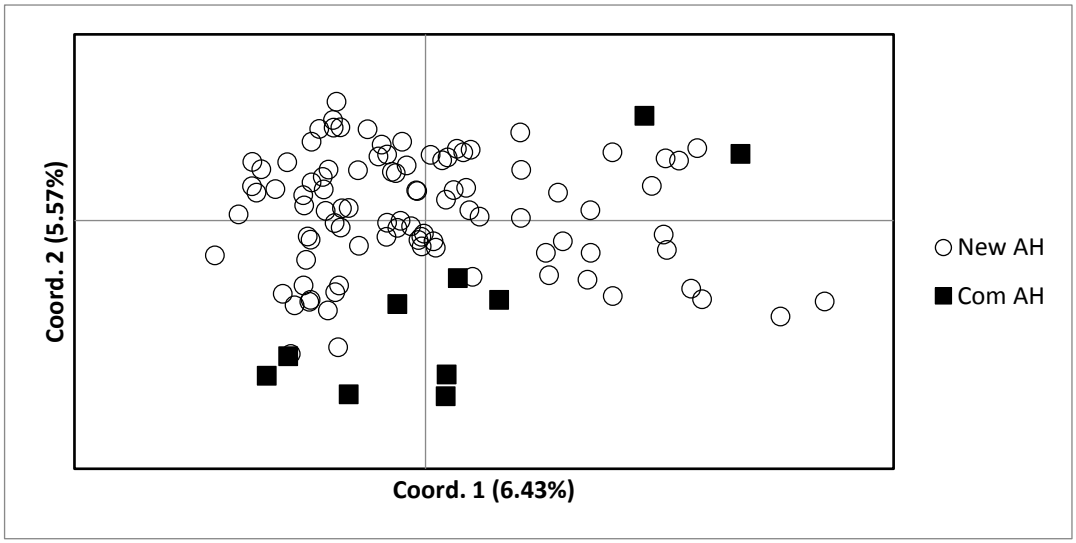


Figure 3.4. Principal Coordinates Analysis based on genotypic distances with data standardisation of hybrid groups (95 newly selected F_1 acacia hybrid clones (New AH) and 10 commercial acacia hybrid clones (Old AH))

3.5. Discussion

It can be difficult to distinguish seedlings of acacia hybrid from those of the parental species based on their morphology (Gan and Liang 1992). The current study demonstrated that assays using a set of 16 SSR markers with analysis using the HIest package is a more reliable way of checking the genetic status of putative acacia hybrid genotypes. We, therefore, recommend the use of this SSR marker set for acacia hybrid identification in breeding programs. Microsatellite markers have been widely and successfully applied for parentage identification, hybrid identification, and purity status testing in some crop plant species, for example cotton and maize (Asif et al. 2009). Our study also illustrates the successful application of the markers in clonal identification, checking the status of clones used to establish pure-species clonal seed orchards, confirmation of pedigrees and monitoring of genetic diversity in acacia breeding programs.

3.5.1. Power of HIest package and newly developed markers for hybridity classification

The results of the simulation study showed that HIest performed better than STRUCTURE in hybrid detection as F_2's could be clearly differentiated from F_1's (100% accuracy) and first backcross generations (96% accuracy) whereas STRUCTURE faced problems in discriminating F_2's from other generations (Le et al. 2016; Vaha and Primmer 2006). HIest was designed specifically for differentiating F_1's, F_2's, backcross and pure species individuals occurring within a single geographic area, whereas STRUCTURE is used for population studies (Fitzpatrick 2012). Plotting ancestry (S) against inter-species heterozygosity (HI) on a triangle-plot (Fig. 3.3) provides a more complete picture of the genetic structure of hybrid populations than STRUCTURE and other methods that use only hybrid index (Fitzpatrick 2012).

3.5.2. Checking the hybridity of Acacia seedlings

One hundred and sixty candidate acacia hybrid clones selected based on morphological criteria were evaluated using the set of 16 SSR markers. Selection based on morphology worked relatively well with seedlings from *A. mangium* mothers (92% were classified accurately as F_1 hybrids thus 8% misclassification). However, in our study morphological assessment did not perform well when applied to selecting hybrid seedlings from *A. auriculiformis* mothers, with only 21% of selections based on morphology classified accurately as F_1 hybrid (79% misclassification). The likely reasons for the poor success rate with seedlings from *A. auriculiformis* mothers are that (i) most of the hybrid candidates from *A. auriculiformis* were identified at age two years, when the seedling leaf characters (Gan and Liang 1992) are no longer available and that (Devey et al.) the assessors who made the selections were not familiar with the wide range of phyllode and other morphological

characteristics present across the natural provenances of pure *A. auriculiformis* (Gan and Liang 1992; Pinyopusarerk et al. 1991).

As expected, all of the ten commercial hybrid clones were confirmed as F_1 hybrids. Most of these clones had been selected in the period 1992-2000, when only *A. mangium* and *A. auriculiformis* were growing in Vietnam. Since then, a total of over 400,000 ha of these ten hybrid clones have been planted throughout the country, with many plantations adjacent or close to most pure species seed orchards, and pollen contamination from hybrid plantations is becoming an issue for pure species breeding programs. The presence of unrecognised F_1 and backcross individuals may create errors in the estimation of genetic parameters from pure-species breeding trials and bias the selection of individuals for further breeding (Harwood et al. 2015). Their presence points to the desirability of establishing isolated seed orchards of both pure species in order to restrict contaminating gene flows.

Microsatellite markers may also be helpful in managing the purity status of planting stocks in seed orchards and detecting hybridisation (contamination) events (Harwood et al. 2015). Approximately 4% of the putatively pure clones in *A. mangium* and *A. auriculiformis* CSOs were found to be hybrids between these species. These hybrids could have arisen from: (1) natural open-pollination in the wild where the two species share the same natural habitat (e.g. Papua New Guinea (PNG) provenances); (2) open-pollination when seed orchards of the two species are near each other; or (3) pollen contamination of pure species seed orchards from adjacent pure-species or F_1 hybrid plantations. It is quite likely that some hybrids came with the open-pollinated seed imported from Australia and PNG. Two natural F_1 acacia hybrids from *A. auriculiformis* and four backcrosses from *A. mangium* mothers were identified from PNG provenances where *A. mangium* and *A. auriculiformis* co-occur, flowering is more or less synchronous and trees of both species are visited by the same pollinators (Sedgley et al. 1992a). We also identified two backcrosses between hybrid and *A. auriculiformis* mothers

that came from a family in SSO in Sakearat, Thailand. These two backcrosses would most likely have arisen from pollination between *A. auriculiformis* in the SSO and natural F_1 hybrid individuals either within or adjacent to the seed orchard.

This set of SSR markers will help the ongoing acacia breeding programs in Vietnam by classifying hybrid generations, enabling more accurate comparison of the relative performance of different categories such as pure species, F_1 and F_2 hybrids and backcrosses between hybrids and the pure species. This can help deployment planning should different hybrid combinations have different adaptive attributes (Harwood et al. 2015).

3.5.3. Clonal confirmation and identification

Microsatellite markers have become a popular tool for verification of cultivar identity because of their abundance, large number of alleles per locus and codominant inheritance, making them highly informative (Garkava-Gustavsson et al. 2008). It is very important to keep track of the genetic identity of operational clones, especially in clonal seed orchard containing superior breeding materials (Suharyanto and Shiraishi 2011). Misidentification may occur at all stages of the process from the development phase to production and planting operations. The economic and genetic impact of any errors in genotype identification will depend on where these occur in the breeding and deployment cycles (Keil and Griffin 1994). In pine, the frequency of incorrectly identified ramets in seed orchards has been estimated to be up to 10% (Wheeler and Jech 1992), resulting in significant loss of genetic gain. The simplest application of a set of SSR markers is fingerprinting, to "determine if two ramets are members of the same or different clones" (Neale et al. 1992) and microsatellite markers are very good for this purpose because of the very low PI that can be achieved, e.g. PI = 1.2×10^{-6} in this study. Microsatellite markers are widely used in forestry to characterise germplasm and for individual identification, e.g. species of the genus *Eucalyptus* (Sumathi

and Yasodha 2014) and *Populus* (Rahman and Rajora 2002). In this study, we observed no errors in samples of paired ramets among 48 randomly selected candidate clones giving confidence that the multiplication process and establishment of the clone bank was carried out to a high standard.

Another potential advantage of SSR markers over traditional, records-based pedigrees is the opportunity to correct pedigree errors, which are common in breeding populations (Adams et al. 1988; Doerksen and Herbinger 2010; Kumar and Richardson 2005; Munoz et al. 2014) due to the multiple steps, complexities of breeding programs and the large number of individuals. Pedigree errors also were found with both controlled crosses (or control pollinated - CP) of loblolly pine (Adams et al. 1988) and open-pollinated (OP) progenies of red spruce (Doerksen and Herbinger 2010) leading to the loss of genetic gain and reduced accuracy of genomic selection. In the present study only one pedigree error was detected. But as the breeding program increases in complexity, more errors are likely to be found (Doerksen and Herbinger 2010).

Ciftci et al. (2017) used microsatellite markers to evaluate the genetic differentiation between clones of European black poplar collected throughout Turkey and found, that nine genotypes that were labelled sometimes differently were represented more than once and one clone was replicated 84 times. This is because these clones are very popular with farmers and breeders. Something similar could easily happen with tropical acacias in the future since they are easy to clone and in Vietnam, for example, only 10 clones have been planted over half a million hectares. In this study, we identified the genotype of these 10 clones (available at http://eprints.utas.edu.au/) so that this can be used for identity check in the future.

Microsatellite markers also have been applied in the management of clone accessions in breeding programs by providing information on the relatedness and DNA fingerprint of

various clones in hybrid poplars (Bekkaoui et al. 2003). In our study, the DNA profiles of 95 confirmed hybrid clones were determined. Each clone could be discriminated using the set of 16 SSR markers. These DNA fingerprinting data could be used for registration of elite clones or for Plant Breeder's Rights protection.

3.5.4. Confirmation of pedigrees and monitoring genetic diversity

The uses of molecular markers to confirm pedigree information can enhance the effectiveness of selection in breeding programs (Jones et al. 2006). The determination of genetic structure and relationships within germplasm collections is also important for breeding (Odong et al. 2011). In cotton, 13 core SSR markers were used to discriminate among accessions as well as group varieties and hybrids of upland cotton based on their origin (Ahmed et al. 2013). Microsatellite markers also have been used in inter-individual genetic distances estimation in *E. grandis* breeding populations, where 97% of 18,336 pairwise distances (with 192 trees) were greater than 0.6 (Kirst et al. 2005). In this study, we found that, as expected, the genetic distances between families were greater than between sibling clones within open-pollinated families of both *A. mangium* and *A. auriculiformis*, with a similar result for control-pollinated F_1 hybrid families. The consistency between genetic distances as indicated by the markers and relatedness according to pedigree records shows that the markers used here can provide an accurate and efficient tool for estimating genetic distances and relatedness among clones. Molecular measures of genetic distance could be used in situations when pedigree information is not available, for example, when selecting candidate clones sourced from unpedigreed SPAs. For example, SSR profiles of teak *(Tectona grandis)* clones enabled separation of clones from different regions of natural occurrence (India and Myanmar) and the natural origin of clones selected in exotic locations such as Nigeria and China to be determined with confidence (Huang et al. 2016).

Experience in Brazil with hybrid eucalypt breeding indicated that the likelihood of finding resistance to pests and diseases is determined more by the genetic diversity in the hybrid combinations under test than by the total number of clones tested (Dehon et al. 2013). Thus, breeding programs for pest/disease resistance of *A. mangium*, *A. auriculiformis* and their hybrid also need to take into account not just the number of clones, but the levels of genetic diversity in the material tested. The new hybrid clones displayed higher genetic diversity than the set of current commercial clones, increasing the likelihood of their containing additional genetic variation in economic traits such as disease tolerance.

Using molecular marker data to assess the diversity of breeding population (including elite populations and seed orchards) in comparison with natural populations can enhance effectiveness of breeding and conservation programs of *Eucalyptus globulus* (Jones et al. 2006). Microsatellite markers (SSR) have been used to analyse the genetic diversity of *E. grandis* seed sources in Kenya in order to support evaluation and genetic conservation (Okun et al. 2008). In the present study, the genetic diversity of the 2001 CSOs of *A. mangium* and *A. auriculiformis* were evaluated indicating they were genetically diverse (H_o= 0.41 in A. *auriculiformis* and H_o= 0.36 in *A. mangium* CSO, data not shown). However, a comparison with a previous study using the same set of SSR markers with native provenances of these two pure species (Le et al. 2016) clearly demonstrated a reduction in genetic diversity in CSOs compared to that in a wide sample of natural provenances (Ho= 0.55 in *A. auriculiformis* and 0.50 in *A. mangium*). Thus, there is scope for using the SSR markers as a guide for infusing new genetic material into the Vietnamese acacia breeding populations.

3.6. Conclusion

This study illustrates the successful use of SSR markers in support of tree breeding, through a case study of acacia in Vietnam. The markers were effective for (1) clonal confirmation

and identification; (2) determining whether individuals were pure-species, F_1 or F_2 hybrids or backcrosses (3) checking pedigrees by comparing observed versus expected relatedness and (4) monitoring levels of genetic variation in selected populations.

Chapter 4 . Patterns of hybrid seed production in adjacent seed orchards of *Acacia auriculiformis* and *A. mangium* in Vietnam

4.1. Abstract

Acacia auriculiformis, *A. mangium* and selected clones of their interspecific hybrid are planted widely in Vietnam. Most new hybrid genotypes are obtained via open pollination between *A. auriculiformis* and *A. mangium*. We examined patterns of hybrid production in adjacent clonal seed orchards of *A. auriculiformis* and *A. mangium* in southern Vietnam by assessing the frequency of hybrid offspring in seed collected from a total of 72 trees at varying distances from the boundary between the orchards. *A. auriculiformis* and *A. mangium* were represented by 17 and 18 clones, respectively, with 2-3 ramets per clone sampled at distances ranging from 4 to 144 m from the species boundary. On average, seventy-five seedling offspring per tree (5,400 seedlings in total) were screened with four species-diagnostic SSR markers (where species have different alleles) using a pooling strategy in which equal proportions of phyllode material from 10 seedlings were combined for DNA extraction. The proportion of hybrid seedlings in each pooled sample was determined from allele peak sizes using calibrations developed from pools with known proportions of pure-species and hybrid material. Hybrid frequency did not differ significantly between the two species, but there were significant ($P<0.001$) differences among individual clones. Differences in flowering phenology appeared to affect hybrid frequency. *A. auriculiformis* flowered slightly later than *A. mangium* and two late-flowering clones of *A. auriculiformis* yielded no hybrid offspring. The level of interspecific hybridisation declined significantly ($P<0.001$) with increasing distance from the species boundary. Trees located within 16 m of the species boundary yielded an average of 9%

hybrid progeny, with the proportion of hybrid seedlings declining to less than 1% for trees over 86 m from the boundary. Trees located more than 112 m from the boundary produced no hybrids. The inferred decline in pollen flow with increasing distance provides guidance for the design of hybridising orchards and isolation requirements to prevent contamination of seed orchards by external pollen sources.

4.2. Introduction

Acacia auriculiformis and *A. mangium*, which are native to northern Australia, New Guinea and adjacent Papua Province in Indonesia, were introduced to Vietnam in the 1960s. Together with their interspecific hybrid (hereafter called acacia hybrid), these have become the most important forest plantation species in Vietnam (Nambiar and Harwood 2014). Breeding programs for these two species have been implemented in Vietnam since the mid-1990s (Hai et al. 2015; Hai et al. 2008; Harwood et al. 2015). The targeted traits for improvement of the breeding programs have been growth, wood properties and stem straightness. Recently, disease resistance has also been addressed (Harwood et al. 2015). Seed production areas (SPAs), seedling seed orchards (SSOs) and clonal seed orchards (CSOs) have been established for improved seed production.

Hybridisation between *A. auriculiformis* and *A. mangium* has been recorded in natural forests and plantations (Kha 2001; Sedgley et al. 1992a). Selected acacia hybrid clones are important for wood production because of their high growth rate and wide adaptability to various environments. Vietnam's acacia hybrid plantation area exceeded 0.5M ha by 2014 (Nambiar et al. 2015), but most of this is from fewer than 10 clones (Kha et al. 2012). Therefore, development of a broader base of acacia hybrid clones is necessary to enhance the productivity and biosafety of this large plantation estate.

Acacia hybrids can be produced via controlled pollination. However, because acacia flowers are small *and* difficult to emasculate it requires much time and expense to produce hybrid seeds (Nghiem et al. 2016). Accordingly, accessing new acacia hybrid genotypes has relied mainly on detection of hybrid individuals within open pollinated progenies of the parental species. Hybrid seed production is most likely in situations where adjacent trees of the two species flower synchronously (Wickneswari and Norwati 1992). However, the effect of distance and genotype is not well understood.

Pollen contamination from unimproved populations and other taxa can decrease the expected genetic gains from breeding (White et al. 2007). In pure-species breeding populations and seed orchards of *A. auriculiformis* and *A. mangium, it* is desirable to minimise contamination from nearby routine acacia plantings, both of the same species and of different species or hybrids. It is therefore important to understand patterns of pollen movement, both for design of plantings for hybrid seed production and isolation of pure-species seed orchards from contamination.

As part of Vietnam's acacia breeding programs, paired clonal seed orchards of *A. mangium* and *A. auriculiformis* were established in 2001 in southern Vietnam (Harwood et al. 2015; Le et al. 2017). The main objective was to produce both pure-species and acacia hybrid seed for testing in the breeding programs. Putative hybrids have usually been identified by morphology assessment at the nursery stage (Gan and Liang 1992) despite this resulting in an over-estimation of hybrid occurrence (Gan and Liang 1992; Le et al. 2017). Recently, the use of species-diagnostic markers, developed by Le et al. (2016), provides a more accurate method of identifying or checking hybrid individuals (Le et al. 2017).

Acacia auriculiformis and *A. mangium* are insect-pollinated species and preferentially outcrossing (Butcher et al. 2004; Moran et al. 1989b; Sedgley et al. 1992b). Pollinator behaviour thus contributes to the pattern of pollen dispersal. In their natural environment,

bees of the genus *Trigona* are the most numerous insect visitors to *A. mangium* flowers and had the highest numbers of acacia polyads on their hairy bodies (Sedgley et al. 1992a; Sedgley et al. 1992b). Honeybees were observed to be the main pollinators of *A. auriculiformis* and *A. mangium* when they were planted together in a hybridising orchard in Vietnam (Nghiem et al. 2011). For bee-pollinated plants, the cross- pollination is usually found at close distances (less than 40 m), although the nature of pollinator behaviour means that occasional pollen grains may travel much further (up to 3.2 km) (Burczyk et al. 2004; Dick et al. 2003; Jha and Dick 2010).

Understanding pollen dispersal has important implications for breeding operations of pure species and acacia hybrid. Pollen dispersal parameters can be directly estimated through paternity analysis using genetic markers. Using 12 microsatellite markers to assess pollen dispersal in two seedling seed orchards of *A. mangium* in Indonesia, Yuskianati and Isoda (2013) concluded that approximately 80% of all crosses were between trees separated by 40 m or less. In a parentage analysis of an *A. mangium* seed orchard in Indonesia, Nurtjahjaningsih et al. (2016) found that pollinations occurred over distances ranging between 15 and 150 m. Pollen dispersal patterns and levels of genetic contamination estimated by microsatellite markers revealed that hybridisation between native populations of *Acacia* subsp. *saligna* and subsp. *lindleyi* occurred at short distances (<300 m); however, occasional hybridisation was also detected over longer distances of around 1600 m (Millar and Byrne 2007; Millar et al. 2008; Millar et al. 2012; Millar et al. 2014). In some other *Acacia* species, longer distances of pollen movement have been recorded. For example, maximum pollinator dispersal distances exceeded 1870 m in *A. woodmaniorum*, a species that occurs in small, isolated populations in dryland Western Australia (Millar et al. 2014).

The aim of this study was to investigate patterns of hybrid seed production in adjacent clonal seed orchards of *A. auriculiformis* and *A. mangium* in southern Vietnam. Factors likely to

influence the proportion of hybrid seed produced, including species and genotype effects, flowering phenology and distance of individual trees from the species boundary, were examined. Hybridisation rates were estimated using species diagnostic SSR markers. The results obtained enabled pollen dispersal across the species boundary between the orchards to be estimated. Practical applications of the finding to hybrid seed production and seed orchard management are considered.

4.3. Materials and methods

4.3.1. Plant material

Adjacent clonal seed orchards (CSO) of *A. auriculiformis* and *A. mangium* were planted in 2001 at Bau Bang (Vietnam). These are surrounded by plantations of cashew nut and rubber for approximately 100 m and Acacias beyond that. Thus the closest acacia plantations are 100 m away. The orchards included 120 clones of *A. auriculiformis* and 100 clones of *A. mangium*, with each orchard having 8 replicates of 2-tree plots of each clone and initial spacing of 4 m x 2 m between trees. Thinning, conducted in 2006 to promote canopy development and high levels of seed production, removed one tree in each two-tree plot and completely removed 20 clones of *A. auriculiformis* (Appendix 4.2).

Open pollinated seedlots were collected from the orchards in 2009 and stored at the Institute of Forest Tree Improvement and Biotechnology (Vietnamese Academy of Forest Sciences - VAFS). From the seed still available in 2016, 72 individual-tree seedlots were selected for the study. Seventeen clones of *A. auriculiformis* were represented by seedlots from two or three ramets per clone and 18 clones of *A. mangium* by two ramets per clone. For each clone, one ramet close to the species boundary and one ramet as far as possible from the boundary was selected. Clones 14 and 1f of *A. auriculiformis* were both represented by three ramets.

Clones in these seed orchards had previously been fingerprinted using SSR markers (Le et al. 2017) and most clones were found to be true to the parental species, but four clones of each species were found to be hybrids (either F_1 or backcross). Locations of the 16 hybrid trees remaining at the time of seed collection are shown in Appendix 4.2. We avoided sampling trees adjacent (more than 10m away) to these known hybrid individuals.

4.3.2. Phenology assessment

Assessment of flowering phenology was carried out in 2015-2016 to provide an estimate of the degree of flowering overlap within and between the two species. The *A. auriculiformis* orchard had been retained unchanged from 2009 and the 17 clones selected for this study were assessed. The number of remaining ramets per clone ranged from 5 to 8. The *A. mangium* orchard had been cut down in 2009 so we were unable to collect phenological data from the 18 *A. mangium* clones. However, progenies of most of the clones in the *A. mangium* CSO were re-tested at the same site in a progeny trial planted in 2009. Phenology data were collected from the progenies of 16 of the *A. mangium* clones, assessing two sibs from each clone. Flowering time and flowering intensity of *A. mangium* and *A. auriculiformis* were recorded fortnightly from October 2015 to the end of January 2016. The flowering intensity of each tree was scored visually using the following categories: (0) - no flowering; light (1) - up to 1/3 of the crown bearing open flowers; moderate (2) - from 1/3 to 2/3 of the crown bearing open flowers; and heavy (3) - more than 2/3 of the crown bearing open flowers. To obtain the monthly flowering intensity of each clone, the scores of all ramets for a particular month were averaged. For *A. mangium*, differences in flowering phenology between progenies of the different clones were not evident, so a single monthly average was calculated from all trees surveyed.

4.3.3. Seed germination

At least 80 seeds per seedlot were nicked at the distal end of each seed and germinated at room temperature in petri dishes containing two layers of filter paper moistened with tap water. Germination was approximately 90% and germinants were transplanted to plastic pots (two plants of the same seedlot per pot) that contained a mix consisting of seven parts composted fine pine bark and 4 parts coarse washed river sand and watered daily.

4.3.4. DNA isolation and pooling strategy

In order to reduce the labour and cost of genotyping, seedlings from the same seedlot were pooled. Each pool included equal quantities of phyllode material from 10 individual seedlings at the same development stage (approximately 10 mg/seedling). Tissue was crushed to a powder in liquid nitrogen and then the standard Qiagen protocol used for DNA extraction (www.qiagen.com/handbooks). DNA concentration and purity were assessed using gel electrophoresis and by comparison with Lambda *Hin*d III molecular weight standard. In total, DNA was isolated from 540 pools (5,400 seedlings). The number of seedlings per seedlot was 75, tested as 7 pools of 10 seedlings with the five remaining seedlings combined with five seedlings derived from another ramet of the same clone. If hybrids were detected in the combined pool, supplementary runs of five seedlings from each maternal ramet were conducted.

4.3.5. PCR conditions and PCR product analysis

PCRs was conducted for all 540 pooled DNA samples using a GeneAmp PCR system 9700 (Applied Biosystems, USA) with a final volume of 12.5µl, consisting of 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM of each forward and reverse primer, 0.5UTaq DNA

polymerase (Invitrogen, Massachusetts, USA) and 20 ng of genomic pooled DNA. The primers from the 6 species diagnostic SSR markers recently developed (Le et al. 2016) were tagged with fluorescent dyes NED, 6-FAM, HEX and ROX on their forward primers and multiplexed. The loci were amplified using a series of touchdown programs with annealing temperatures (T_a) spanning 10^0C. The annealing temperature range was centred on the average optimal T_a for the six pairs of primers. The touchdown program initial annealing temperature was 60^0C, which decreased to 50^0C over 20 cycles, achieved by decreasing the temperature by 0.5^0C every cycle. This was followed by a further 20 cycles using a 50^0C annealing temperature. Cycling was composed of a 95^0C hold for 1 min, annealing temperature for 1 min and 72^0C for 1 min. Cycling was preceded by a hold at 95^0C for 5 min to provide a "hot start" and finished with a final hold of 10 min at 72^0C.

PCR products were separated using an ABI 3730 DNA Analyzer (Applied Biosystems, USA) by the Australian Genome Research Facility (http://www.agrf.org.au). Raw data were analysed using GeneMapper 3.7 (ABI, USA) and Geneious R10 (HTTP:// www.geneious.com) software and peak size for each possible allele were recorded (see below for further explanation).

4.3.6. Test of pooling strategy

We tested the ability of using our pooling technique in combination with the species-diagnostic markers developed by Le et al. (2016) to determine the frequency of hybrids between *A. auriculiformis* and *A. mangium*. We used 50 pure *A. auriculiformis* (AA), 50 pure *A. mangium* (AM) and 25 control pollinated F_1 hybrid (AH) seedlings to construct 13 types of pools of 10 samples having various contributions of alleles from each species (Table 4.1).

Table 4.1. Species contributions for the 13 pools used to test the pooling strategy, with expected mean peak ratio for the *A. mangium* (AM) specific allele for each pool type, and peak ranges used for assigning numbers of hybrid (AH) seedlings. *Acacia auriculiformis* is abbreviated as AA

Pool type	Ratio of species-specific alleles	Expected mean peak ratio for *A. mangium*-specific alleles	Peak ratio range (q value) for assigning no. of hybrid seedlings
10AA	20AA:0AM	0	0 – 0.004
9AA:1AH	19AA:1AM	0.05	0.005 – 0.074
8AA:2AH	18AA :2AM	0.10	0.075 – 0.124
7 AA:3AH	17AA:3AM	0.15	0.125 - 0 0.174
6 AA:4AH	16 AA:4AM	0.20	0.175 – 0.224
5AA:5AH	15AA:5AM	0.25	0.225 – 0.274
10AH	10AA:10AM	0.50	0.475 - 0.524
5AM:5AH	5AA:15AM	0.75	0.725 – 0.774
6AM:4AH	4AA:16AM	0.80	0.775 – 0.824
7AM:3AH	3AA:17AM	0.85	0.825 – 0.874
8AM:2AH	2AA:18AM	0.90	0.875 – 0.924
9AM:1AH	1AA:19AM	0.95	0.925 - 0.995
10AM	0AA:20AM	1	0.996 – 1.0

Each pool type was replicated four times with different sets of seedlings giving a total of 52 test pools. Because the frequency of hybrids was expected to be low in these seed orchards we constructed pools dominated by either pure species and with a low frequency of hybrids (0 to 5 hybrids for each species). The pools were prepared by mixing 10 mg of phyllode tissue from each seedling and DNA from the pooled sample extracted using the Qiagen protocol as explained above.

The proportion of hybrids in a pool was estimated from the ratio of peak sizes due to the diagnostic alleles for each species at each marker. When more than one diagnostic allele per locus was present for a species their peak sizes were summed. The number of hybrid individuals in each pool was predicted by the average peak size ratio for four markers.

4.3.7. Statistical analysis

We used q value to predict the number of hybrid individuals in the pools. The q value was calculated as the mean ratio of diagnostic peak sizes that was obtained from four markers (e.g. q value = peak value of *A. mangium* alleles/ (peak value of *A. mangium* alleles + peak value of *A. auriculiformis* alleles). A pool was predicted to contain one hybrid if q was equal to 0.05 ± 0.025; if q = 0.01 ± 0.025 the pool was predicted to have two hybrids, etc. (Table 4.1). Pools comprising 10 samples from either pure species were expected to yield no detectable peak representing alleles of the other species (q = 0 or 1). To examine the accuracy of hybrid detection with the pooling strategy, we calculated linear regressions of predicted (based on peak ratios) versus expected (based on seedling mix) allele frequencies for each marker in the pool, using the SPSS program (https://www.ibm.com). The predicted versus expected values for all 52 individual pools in the test, based on the mean peak ratios of the four markers which performed satisfactorily, were then compared.

The total number of hybrids in each seedlot was estimated by summing the numbers of predicted hybrids across all pools of that seedlot. To investigate genetic and distance effects on the rate of hybridisation, a linear model was fitted with a number of hybrid individuals (out of 75 progenies tested) per tree as the dependent variable and species and clone within species as factors and distance and distance x species as covariates, using PROC GLIMMIX of SAS (Version 9.4, SAS Institute). A square root transformation of the dependent variable was subsequently used to improve the distribution of residuals. To find the curve which best

described the decay in the number of hybrid with distance, a variety of non-linear models (negative exponential, power, logistic and logarithmic functions) were fitted using the SPSS program (https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software).

4.4. Results

4.4.1. Flowering phenology

Acacia auriculiformis clones flowered later than *A. mangium* over the period from September 2015 to January 2016 (Fig. 4.1). *Acacia mangium* commenced flowering in September and was finished by December, whereas, *A. auriculifomis* flowered from October to January (Fig. 4.1). For *A. auriculiformis*, peak flowering was generally observed in November and December. One period of synchronous flowering with high flowering intensity (moderate to heavy) in both species was observed in November, with the exception of clones 155, 18 and 29 of *A. auriculiformis* which had little overlap in flowering with *A. mangium* in December.

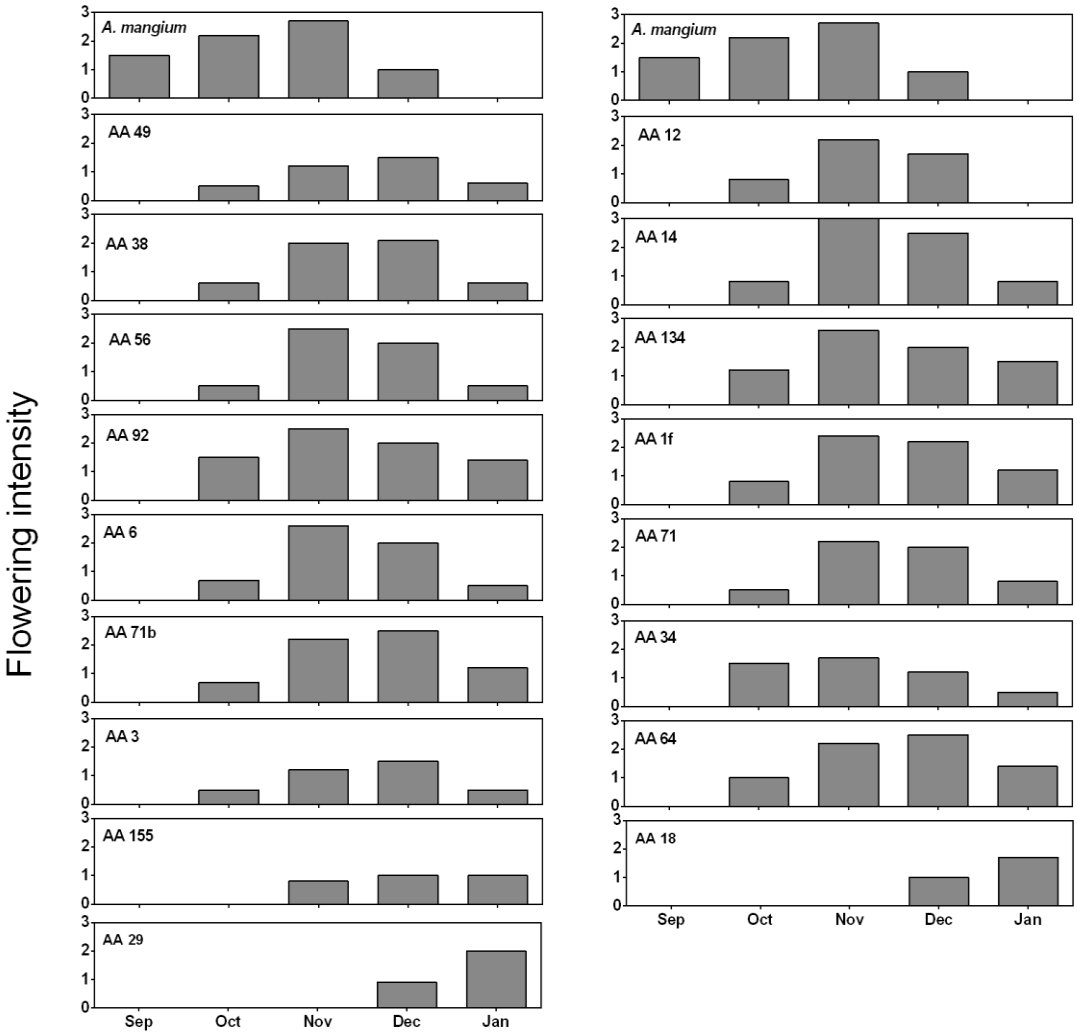


Figure 4.1. Monthly average flowering intensity of *A. auriculiformis* clones in comparison to *A. mangium* (mean of 32 *A. mangium* trees is shown at the top of both columns to facilitate comparison with each *A. auriculiformis* clone)

4.4.2. Validation of the number of hybrid prediction using pool strategy

We evaluated separately each of the six species diagnostic SSRs. Two markers (AH3_17, AH08) had regression results which were inconsistent with expectation, linear regression accounting for less than 60% of the variance in observed versus expected allele peak ratios (Table 4.2) and these were dropped from further analysis. The other four markers showed

better relationships with linear regressions explaining 96 – 99% of the variance. However, approximately 40% of the pools are incorrectly predicted (Table 4.3), but in all cases this is by only one hybrid.

Table 4.2. Percentage (%) of variance explained by linear regression of expected versus observed allele frequencies in pools of 10 seedlings with known proportions of hybrid seedlings pooled with pure-species seedlings of either *A. auriculiformis* or *A. mangium*

Pure species in mix	AH3_6	AH54	Ancp54&55	Acnp16&17	AH08	AH3_17
A. auriculiformis	99	98	98	99	74	49
A. mangium	99	98	.98	96	45	56
Mean	99	98	98	97	59	52

Table 4.3. Predictions of numbers of hybrid individuals in each pool type based on mean allele peak ratios in relation to expected numbers based on seedling frequencies

Pool type	Number of replications	Number of successfully predicted pools	No. of pools under-predicted	No. of pools over-predicted
10AA	4	4		
9AA:1AH	4	3		1
8AA:2AH	4	2		2
7 AA:3AH	4	3		1
6 AA:4AH	4	2		2
5AA:5AH	4	2	1	1
10AH	4	3	1	
5AM:5AH	4			4
6AM:4AH	4	2	2	0
7AM:3AH	4	1		3
8AM:2AH	4	2		2
9AM:1AH	4	3		1
10AM	4	4		
Total	52	31	4	17

4.4.3. Frequency of hybrid progeny's in the sampled trees

Most of the pools had no or few hybrids. Out of 540 pools, there were 414 with no hybrids, 101 with one hybrid, 21 had two hybrids and three pools had three hybrids, one pool had four hybrids, and no pool had five or more hybrids. In total out of 5,400 progenies, 156 were

predicted to be hybrids. However, this is likely to be an overestimate as shown in Table 4.3. We calculated that this over-estimation was around 10%.

The results also showed that *A. mangium* and *A. auriculiformis* yielded similar percentages of hybrid seedlings overall (3.4% and 2.8%, respectively, the species difference was not significant (Table 4.4)). Distance from the species boundary was the most important factor influencing the production of hybrid seed yields ($P < 0.001$, Table 4.4). There was no significant effect of distance by species interaction ($P = 0.6$).

Table 4.4. Univariate analysis of covariance analysis of the number of interspecific hybrid individuals per tree (square root transformed) in adjacent clonal seed orchards of *A. auriculiformis* and *A. mangium*.

Effect	Numerator DF	F value	Probability
Clone	33	3.14	<0.001
Species	1	0.03	0.87
Distance	1	60.8	<0.001
Distance x Species	1	0.27	0.60

The highest frequency of interspecific hybridisation was found close to the species boundary and frequency declined with increasing distance in both species (Fig. 4.2). Trees within 16 m of the species boundary (16 trees/species) yielded on average 9.1 % hybrid progeny. The average level of interspecific hybridisation for trees between 16 m and 64 m of the boundary reduced to 4.5% (mean of 24 trees per species). Trees between 68 m and 112 m from the boundary produced an average of 1.2% hybrids (24 trees), and trees located more than 116 m to species boundary produced no hybrid seeds. Approximately 80% hybrid individuals

were found from mother trees that were located within 52 m from the species boundary (Appendix 4.3).

Clones differed significantly in the production of hybrid seed ($P < 0.001$; Table 4.4). Across both species, 27 out of 72 trees yielded no hybrids and some of these were trees close to the species boundary. Three *A. auriculiformis* and five *A. mangium* clones yielded no hybrids from either of their two ramets (Appendix 4.1). *Acacia auriculiformis* clones 18 and 29, which had the least overlap in flowering with *A. mangium*, and clone 155, which had low flowering intensity and only moderate overlap with *A. mangium*, produced no hybrid seeds.

The overall decline in the hybridisation rate with distance was best modelled by a power function: Number of hybrid = -2.52 ln (distance) + 11.65, $R^2 = 0.67$. There were major departures from the curve, for example, one individual of *A. mangium* 44 m from the species boundary had 13 hybrid offspring among the 75 progenies tested (Fig. 4.2).

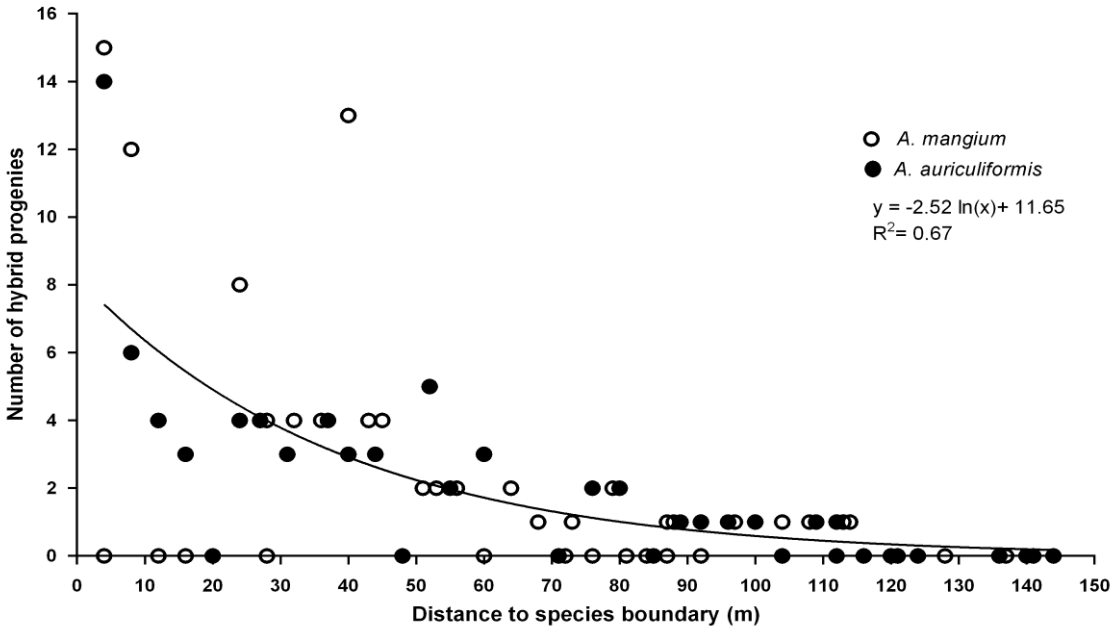


Figure 4.2. Effect of distance on the number of interspecific hybrids (on average) detected among 75 open -pollinated seed collected from *A. auriculiformis* and *A. mangium* trees in adjacent seed orchards (each point in the graph is the result from one tree).

4.5. Discussion

In this study the number of hybrids in samples of open pollinated seed of acacia trees was estimated from quantitative data on species diagnostic allele frequencies obtained from pooled DNA samples of progenies from single trees. Because allele frequencies and hence hybrid frequencies were predicted from relative fluorescence peak heights, the procedure was prone to errors inherent in the PCR process. In this study we estimated that approximately 40% of the pools either overestimated or underestimated the number of hybrids. However, in all cases this error was of a small magnitude (by only one) and the pools most likely to have errors were those containing 2, 3 4, and 5 hybrids which were relatively rare in our study. We calculated that over-all this over-prediction in our study was approximately 10%. Hence, we predicted there were 156 hybrids among the 5,400 seedlings studied, but in fact it is likely that there were 144 hybrids. In the future, if we had the resources, we could go back to the pools with prediction of two, three or four hybrids, and split them into two pools of five samples to more accurately determine the true number of hybrids.

The hybrid and backcross trees present within each species orchard may have produced pollen with hybrid-identifying alleles. We could not account for the contribution of these trees on the estimates of hybrid frequency. However, we consider it would be small, for four reasons. Firstly, because we avoided sampling trees within 10 m of the known hybrid and backcross individuals in each orchard. Secondly, the contribution of species-diagnostic alleles from the backcross and F_1 hybrid trees should be a quarter and half, respectively, of that of a pure-species tree of the other species. Thirdly, the hybrid and backcross trees represent only 2% of the trees in both orchards, and thus would be expected to be male parents to only 2% of the progenies of other trees. These hybrids and backcross trees had similar flowering intensity to the pure species trees. Fourth, we checked whether there was

a correlation between the number of hybrids found in each tree and the closest distance to a hybrid or backcross tree, and found it was not significant (r = 0.13, P = 0.26).

4.5.1. Influence of distance from species boundary on hybrid seed yields

The frequency of hybrids production declined with increasing distance between the species. The results suggest that pollen flow of the two species decreases exponentially with increasing distance from the pollen source, with very little pollen travelling beyond 100 m. At distances of 0-16 m, the rate of hybridisation averaged 9%. The highest rates of individual hybrid occurrence (15/75 progeny in *A. mangium* and 14/75 in *A. auriculiformis*) equates to 19-20% hybrid frequency. This concurs with an earlier study in Malaysia, in which trees were sampled approximately 10 m from the boundary of adjacent plantation blocks of these two species and yields of hybrid progeny ranged from 0.7 to 21.7% (mean 6.9%) in *A. mangium* and 2.9 to 14.7% (mean 9.3%) in *A. auriculiformis* (Wickneswari and Norwati 1992).

In this study, 80% of hybridisation events occurred within 52 m of the species boundary (Fig. 4.3). This was consistent with the cumulative pollen dispersal curves in pure-species *A. mangium* seed orchards in Indonesia (Yuskianti and Isoda 2013) where 80% of cross-pollinations occurred between trees separated by 40 m or less, although occasional dispersal events took place over 100 m or more. Similar results were found in planted stands of *A. saligna* where the average pollen dispersal distance was 37 m with the majority of progeny sired by paternal trees within a 50 m neighbourhood of the maternal trees (Millar et al., 2008). Our results are also consistent with other studies on pollen dispersal in *Eucalyptus* species, which are mainly pollinated by bees with a distance of less than 300 m from external pollen sources (Barbour et al. 2003; Barbour et al. 2005; Barbour et al. 2002).

The movements of pollinators are the main determinant to the hybrid production in this study. This is in part due to the fact that pollinia are presumed to be too large to be effectively dispersed by wind (Sedgley et al. 1992a). In Brazil, honey bees collect *Eucalyptus saligna* pollen of different plants in one visit and were found to promote cross-pollination up to 100 m, decreasing gradually up to a distance of 300 m (Pacheco et al. 1986). Honey bees and native bees are main pollinator of acacias (Nghiem et al. 2011; Sedgley et al. 1992a). In Vietnam, farmers commonly place hives of honey bees in acacia plantations for honey production. The introduction of bee hives within seed orchards has been shown to change the natural pattern of seed production and the number and positioning of hives will affect pollen dispersal patterns (Moncur et al. 1995). In 2009, there were bee hives present within 200 m but not within the orchards.

4.5.2. Effects of phenology and genetic differences

There were substantial differences between clones within species in hybrid yields. The limited overlap in flowering time of some clones of *A. auriculiformis* with *A. mangium* is a likely contributing factor. Fourteen out of 17 *A. auriculiformis* clones had at least one flowering flush that was synchronous with *A. mangium*. Hence, hybrid seedlings were found in all of these clones. The phenological data indicated that two of three *A. auriculiformis* clones (clone 18 and 29) which produced no hybrid offspring flowered later than *A. mangium*. Ramets of the third clone (155) were both distant to the species boundary (84, 140 m) and displayed low flowering intensity and late flowering, which would have reduced opportunities for inter-species pollen flow to this clone.

There were also five *A. mangium* clones that yielded no hybrid seeds. Unfortunately, phenological information on the *A. mangium* clones was not available. Early-flowering clones of *A. mangium* would have had little overlap with *A. auriculiformis.* The more

overlap in flowering time, the greater the chance to find hybridisation in the orchard (Josue 1992). It has been suggested that flowering time is probably the major determinant of the levels of hybridisation between plantations of *Eucalyptus nitens* and adjacent natural stands of *E. ovata* and *E. viminalis*. Hybridisation of *E. nitens* with *E. ovata* have been observed, whereas no hybrids between *E. nitens* and *E. viminalis* have been detected. This was explained simply by the lack of overlap in flowering time between *E. nitens* and *E. viminalis* (Barbour et al. 2002).

Phenology data was collected in 2015 and 2016, 7 years after the seed used here were collected and this could have influenced our results. However, two studies of flowering in *Acacia* species across the years had been conducted and these have found no significant variation among seasons. There were no significant variations among the three seasons in flowering initiation and flowering period among clones of 19 *A. mangium* and 23 *A. auriculiformis* clones (Kato et al. 2012). The same result was also found in *Senegalia senegal* (formerly *Acacia senegal*) (Omondi et al. 2018).

4.5.3. Implications for seed orchard design and management

The similar rate of hybrid production from the two species confirms the lack of reproductive barriers noted in previous studies (Nghiem et al. 2013; Sedgley et al. 1992a; Sedgley et al. 1992b). The hybrid yields would be higher if more trees overlap in flowering time. Flowering time has been found to be highly heritable in *Eucalyptus globulus* (Jones et al. 2011). If, as is apparent from the study of Kato et al. (2012), the same is true in these tropical *Acacia* species, it would be possible to select clones of both pure species to achieve greater overlap in their flowering time. However, this would reduce diversity in the breeding populations for hybrid generation. Considering planting designs to promote hybridisation, alternating single rows of *A. auriculiformis* and *A. mangium*, at sufficiently wide spacing to

promote canopy development of both species, would seem likely to maximise the proportion of hybrid seed produced. Use of a wide range of genotypes seems advisable, rather than using a limited set of genotypes of *A. auriculiformis* and *A. mangium*, some of which might not overlap in their flowering times. By monitoring flowering time in such seed orchards, the genotypes that overlap the most in flowering time can be identified. Seed collection and hybrid detection can target those trees and maximise the chance of identifying hybrids. Placing beehives in seed orchards when flowering of the two species overlaps is also recommended to promote pollen movement between the species (Moncur et al. 1995). Controlled pollination using stored pollen of early-flowering *A. mangium* genotypes to late-flowering *A. auriculiformis* genotypes is also an option for hybrid production, although technically demanding and expensive.

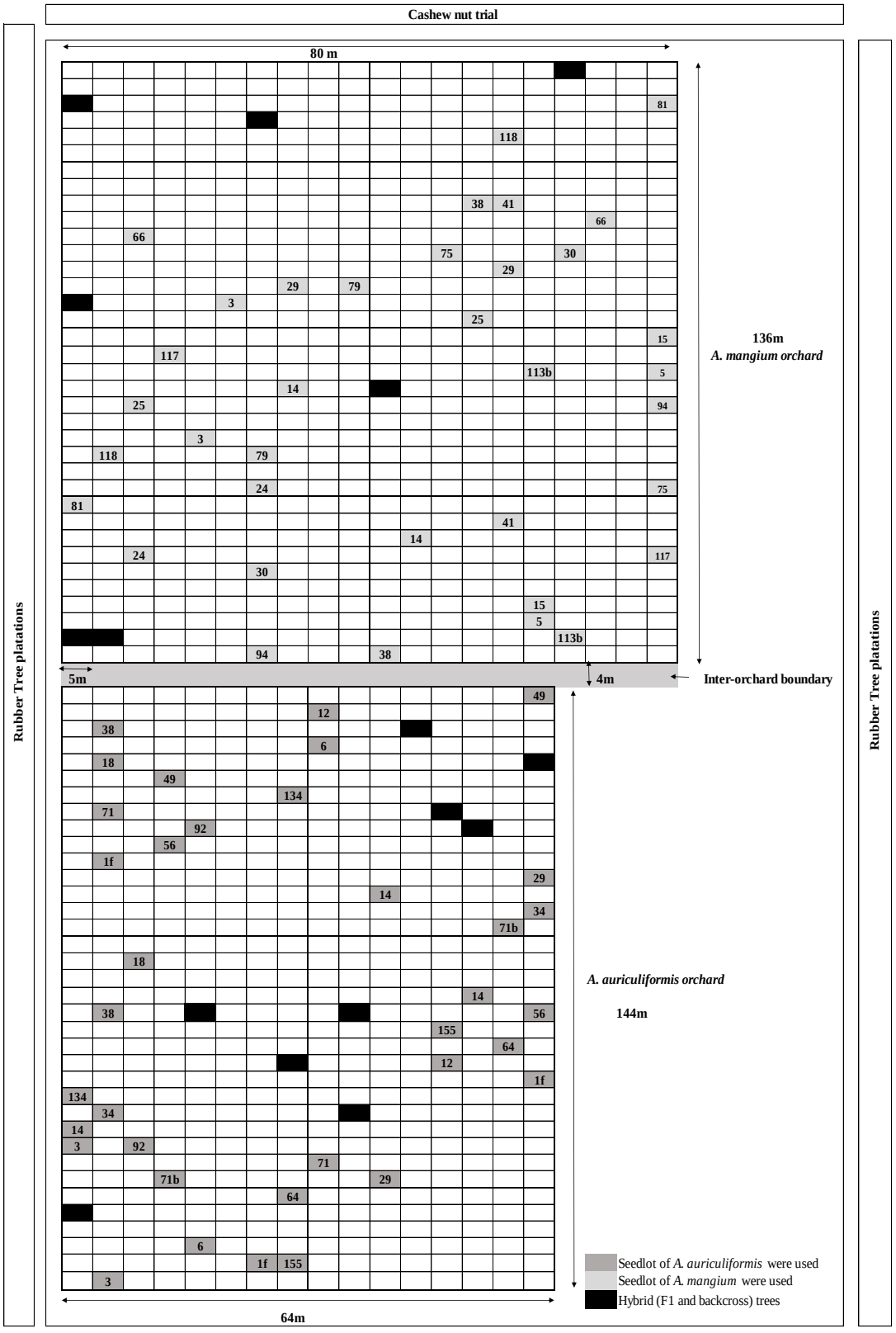
The acceptable level of external contamination in seed orchards and breeding populations depends on the purpose of seed production. For production orchards of *A. mangium* and *A. auriculiformis*, levels of about 3%, the orchard averages detected in the current study, would be acceptable as such levels would not lower genetic gain and hence plantation productivity appreciably, even if the seedlings derived from the external pollen source performed poorly (White et al. 2007). We studied two orchards, each approximately square in their layout and slightly over 1 ha in area and each with a potential contamination source contiguous to one of their four borders. Mean levels of contamination would have been higher if they had each been surrounded by potential sources of contamination on all four of their borders. Orchard size is another determining factor of contamination levels since, other things being equal, increasing orchard size above 4 ha should yield an increasing proportion of orchard trees further than 100 m from the nearest orchard boundary, which on the basis of our study should receive very low levels of contamination. Contamination is a more critical issue for breeding orchards than for production orchards, since the default assumption for accurate estimates

of genetic parameters and accurate selection of individuals for further breeding is that no offspring from the breeding orchard will have external pollen parents. The levels of contamination approaching 10% observed in trees close to the inter-orchard edge in this study would be unacceptably high for breeding purposes. Around some orchards in Vietnam, buffer rows of *Eucalyptus* have been planted to reduce pollen contamination from external pollen sources. The effectiveness of this measure has not yet been quantified. This study identified that a separation of 116 m was enough to prevent any hybridisation from an interspecific contaminating sources. The flowering phenology of the orchard genotypes and potential contaminants will influence contamination — pollen transfer from synchronously flowering trees of the same species would probably be higher than what was found in the current study. The separation distance to prevent contamination will also vary *d*epending on factors such as bee management and pollen source/sink intensity (Dick et al. 2003; Richards 1997). Nonetheless, our results strongly suggest that an isolation distance of 100 m from nearby potential pollen parents would greatly reduce contamination of *A. auriculiformis* and *A. mangium* orchards.

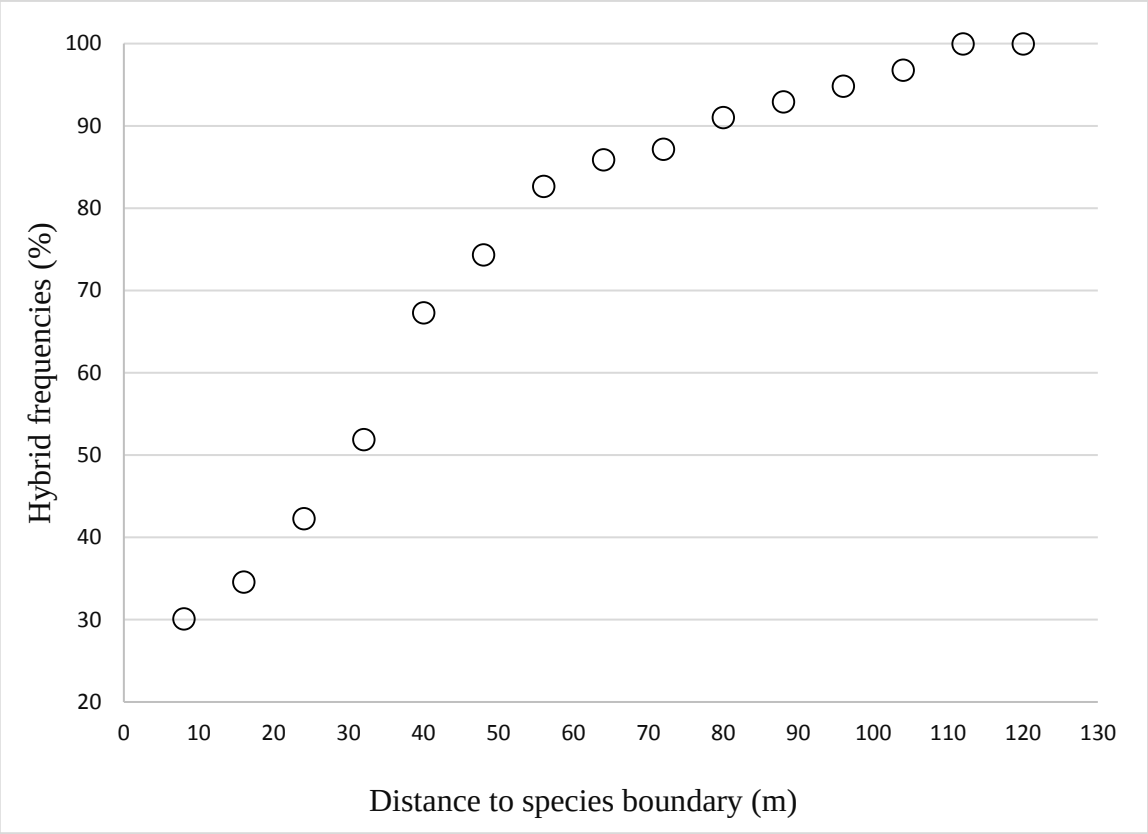
Appendix 4.1. Distance from inter-orchard boundary and number of hybrid progeny from 75 germinated seeds for each tested ramet of each clone of *A. auriculiformis* (AA) and *A. mangium* (AM). The percentage (%) of hybrid progeny for each clone is also shown.

Species	Clone ID	Distance from inter-orchard boundary (m)			Number of hybrid progeny			Clone % hybrids
		Ramet No.	1	2	3	1	2	3	
AA	49		4	24		14	4		12.0
AA	12		8	92		6	1		4.7
AA	38		12	80		4	2		4.0
AA	14		52	76	108	5	2	1	3.6
AA	56		40	80		3	2		3.3
AA	134		28	100		4	1		3.3
AA	92		36	112		4	0		2.7
AA	6		16	136		3	0		2.0
AA	71		32	116		3	0		2.0
AA	71b		60	120		3	0		2.0
AA	1f		44	96	140	3	1	0	1.8
AA	34		56	104		2	0		1.3
AA	3		112	144		1	0		0.7
AA	64		88	124		1	0		0.7
AA	18		20	72		0	0		0.0
AA	29		48	120		0	0		0.0
AA	155		84	140		0	0		0.0
AM	38		4	112		15	1		10.7
AM	81		40	136		13	0		8.7
AM	113		8	72		12	1		8.7
AM	30		24	100		8	1		6.0
AM	24		28	44		4	4		5.3
AM	14		32	68		4	1		3.3
AM	41		36	112		4	1		3.3
AM	75		44	96		4	1		3.3
AM	3		56	88		2	1		2.0
AM	79		52	88		2	1		2.0
AM	66		104	108		1	1		1.3
AM	94		4	64		0	2		1.3
AM	118		52	128		2	0		1.3
AM	5		12	72		0	0		0.0
AM	15		16	80		0	0		0.0
AM	25		60	84		0	0		0.0
AM	29		88	92		0	0		0.0
AM	117		28	76		0	0		0.0

Appendix 4.2. Layout of the paired clonal seed orchards of *A. auriculiformis* and *A. mangium* at Bau Bang, South Vietnam showing positions of seed trees that provided progenies for assay of hybrid frequency



Appendix 4.3. The cumulative frequency of hybrid individuals as distance from the species boundary increases



Chapter 5 Reproductive and growth characteristics of allotetraploid acacia hybrid (*Acacia mangium x A. auriculiformis*)

5.1. Abstract

Acacia hybrid (*Acacia mangium x A. auriculiformis*), typically planted clonally, has become one of the most important planted tree taxa in Vietnam due to high productivity, adaptability and commercial wood yields. Polyploid breeding offers further possibilities for enhancing hybrid vigour. Colchicine-induced tetraploid lines derived from three commercially-grown diploid acacia hybrid clones were grown together with diploid progenitors in a field trial. In this study, we compared the flowering time, outcrossing rate, seed size and germination of these allotetraploid and diploid clones, as well as inbreeding depression and marker inheritance of their offspring in order to understand better how to manage acacia hybrid breeding and deployment populations.

Tetraploid acacia hybrid flowered slightly later but more intensely than the diploid cytotypes. Tetraploids produced the same number of seeds per pod but larger seeds than their diploid progenitors. The outcrossing rate of tetraploid acacia hybrid lines (14%) was very low compared to their diploid progenitors (87%). By investigating the inheritance of molecular markers in outcrossed progenies, it can be concluded that the tetraploid acacia hybrid has intermediate or mix model inheritance (with both disomic and tetrasomic marker inheritance) and it thus appears to behave as a segmental allotetraploid. The inbreeding depression in growth at 12 months of age for self compared to outcross progenies was greater for the diploid cytotype (33%) than for the tetraploid cytotypes.

5.2. Introduction

Acacia mangium, *A. auriculiformis* and their natural interspecies F_1 hybrid (referred to subsequently as acacia hybrid) are important for wood production in SE Asia due to high growth rates, acceptable wood properties and high adaptability to various tropical environments (Harwood et al. 2015; Kha 2001). There is an estimated 2 M ha of tropical acacia plantations in Indonesia, Malaysia and Vietnam (Harwood et al. 2015). In Vietnam, acacia hybrid clones are widely planted and approximately 0.5 M ha of this taxon had been established by 2014; a similar plantation area to that of *A. mangium* (Nambiar et al. 2015). Breeding programs for *A. mangium*, *A. auriculiformis* and acacia hybrid were established in Vietnam in the 1990s. Deployment in plantations is either clonal (for acacia hybrid and *A. auriculiformis*) or from open-pollinated seed from seed orchards (for *A. mangium*) (Harwood et al. 2015). Polyploid breeding of acacia commenced in Vietnam in 2003 with the main aim of producing superior triploid (*3x*) genotypes (Griffin et al. 2015). The production and use of triploids are being investigated in acacia as a way of reducing invasiveness (Griffin et al. 2015). Griffin et al. (2015) proposed an open-pollinated synthetic hybrid breeding strategy for polyploid acacias with prospects of improving productivity, pest and disease resistance and ease of clonal proparation, but the sucees of this strategy depends on the breeding system. However, we know very little about the biology and breeding system of advanced generation acacia hybrids.

Breeding from neo-allotetraploids (*4x*), which are commonly generated by hybridising two different diploid (*2x*) species and doubling their genome (Soltis and Soltis 2012), is considered as a possible strategy for producing highly heterozygous advanced generation acacia hybrids. This is because allotetraploids may experience less intergenomic recombination than autotetraploid, therefore, effectively maintaining the same level of heterozygosity through the generations as found in F_1 hybrids (Griffin et al. 2015; Mendoza

and Haynes 1974). In interspecific hybrids, fixed heterozygosity is associated with hybrid vigour and increases in traits such as growth, size of harvested organs such as stem, fruit, seeds and reproductive outputs (Leal-Bertioli et al. 2017; te Beest et al. 2012). However, not all allotetraploids experience such hybrid vigour (Leal-Bertioli et al. 2015; Leal-Bertioli et al. 2017) and in some cases, this may be due to chromosomal instability (Henry et al. 2014; Mason and Batley 2015).

In theory, there are two extremes in tetraploid inheritance, disomic in allotetraploid (where bivalents are formed at metaphase I during meiosis) and tetrasomic in autotetraploid (where quadrivalent are formed at meiosis) (Lloyd and Bomblies 2016; Stift et al. 2008). Disomic inheritance has been suggested to lead to stability in chromosome number hence fertility, while tetrasomic inheritance leads to instability in chromosome number, recombination between genomes and infertility (Lloyd and Bomblies 2016; Ramsey and Schemske 2002). Tetrasomic inheritance in new allotetraploids may be more common than once considered and it has been suggested that many allotetraploids may, in fact, be 'segmental allotetraploid' with a mixture of disomic and tetrasomic inheritance (Leal-Bertioli et al. 2015).

While the effect of polyploidy on chromosome stability has received much scientific attention, relatively less attention has been given to the effect of polyploidy on breeding system. Studies of breeding systems of trees, including outcrossing rates, are important because inbred progenies are generally expected to suffer from inbreeding depression (Barringer and Geber 2008). Inbreeding depression is the reduction in biological fitness of offspring derived from inbreeding events compared to outcrossed offspring (Husband and Schemske 1996). Inbreeding depression is a major negative consequence of selfing (Rausch et al. 2005). The expected selfing rates in polyploids are generally higher than that in their related diploid progenitors (Ozimec and Husband 2011) due to the breakdown of self-incompatibility systems (Miller and Venable 2000). The breeding system of natural

populations of diploid *A. auriculiformis, A. mangium* and in the diploid F_1 hybrid is predominantly outcrossing (approximately 86% for all taxa) with some exceptions for populations of pure species on the edge of their natural distribution which tend to have low outcrossing rates and low allelic diversity (Butcher et al. 2004; Moran et al. 1989b; Ng et al. 2009). These diploid taxa produce a large number of seed per pod from open-pollination (6.2 seed/pod *A. auriculiformis,* 8.3 in *A. mangium*, (Nghiem et al. 2016) and from 4 to 13 in acacia hybrid (Ng et al. 2009)). In diploid *A. mangium*, seedlings derived from open-pollinated families with a high degree of selfing had poorer growth performance compared to seedlings from families with high degree of outcrossing (Harwood et al. 2004).

The reproductive biology and growth characteristics of autotetraploid *A. mangium* were examined by Nghiem et al. (2011). There was no evidence of barriers to cross-pollination and fertilisation between *4x* and *2x* cytotypes. However, pod set and the number of full seeds per pod were much reduced in *4x* compared to *2x* (Nghiem et al. 2011). In a study of the breeding system of autotetraploid *A. mangium*, the outcrossing rate averaged only about 2% (98% selfing), whereas the outcrossing rate of diploid trees in the same seed orchard was 97% (Griffin et al. 2012). In a follow-up study of field performance of open-pollinated progenies collected from autotetraploid and diploid *A. mangium,* the autotetraploid progenies showed poor performance in growth in comparison with diploid progenies (Griffin et al. 2015). Griffin et al. (2015) observed that some autotetraploid families showed a high frequency of abnormal genotypes in the field trial.

Colchicine-induced allotetraploid clonal lines from three diploid commercial acacia hybrid clones (Kha et al 2012) have been produced (Griffin et al. 2015; Price et al. 2014). The clones were all confirmed to be true F_1 hybrid based on their allelic profiles for species-specific SSR markers (Le et al. 2017). These allotetraploid clones were planted together with their diploid progenitors in a field trial at Bau Bang in Vietnam, to investigate the

possibility of triploid production by open-pollination. The present study was conducted at that site. We investigated the breeding system (flowering time, seed production, outcrossing rate) of allopolyploid acacias in comparison with their diploid progenitors, and also inbreeding depression and chromosome stability in their progeny. These attributes of diploid and tetraploid acacia hybrids were compared with previously reported behaviour of the diploid and autotetraploid cytotypes of *A. mangium* to provide insights for future breeding and deployment strategies.

5.3. Materials and methods

5.3.1. Germplasm and field trial design

The field trial at Bau Bang included 12 successfully rooted *4x* lines (3-5 per genotype, Table 5.1) as well as the three original *2x* commercial hybrid clones (BV10, BV16 and BV33) and was planted in six replicates. Each replicate contained 3 blocks, each with 16 ramets of *2x* and 16 ramets of *4x* for one of the genotypes, giving a total of 576 trees in the trial, with each *4x* acacia hybrid genotype represented by ramets from its different lines. The spacing between rows was 3 m and the initial spacing between trees within rows was 2 m. The trial was selectively thinned in 2013, with about 50% of trees removed to promote canopy development and flowering, the ratio of *2x* and *4x* ramet were mostly maintained as before the thinning. The trial was surrounded by *A. auriculiformis* and acacia hybrid plantations of flowering ages in close proximity (5 m) to the trial.

5.3.2. Phenology study

In order to assess the potential for open-pollination among the different clones and cytotypes, the flowering of all remaining trees in the trial was scored every two weeks from September

2015 to February 2016 (Table 5.1). The number of ramets per genotype that flowered was counted to estimate the percentage of the flowering trees per genotype and per cytotype. This data was aggregated to give the cumulative number of ramets that flowered every month for each genotype/cytotype combination. The flowering intensity of each tree was scored visually using the following categories: 0 - no flowering, 1 - up to 1/3 of the crown bearing opened flowers, 2 - from 1/3 to 2/3 of the crown bearing opened flowers, and 3 - more than 2/3 of the crown bearing opened flowers. To obtain the monthly flowering intensity of each genotype/cytotype combination, the scores of all ramets, for a particular month, were summed and divided by the total number of ramets and then divided by 3 (the highest intensity) and expressed as the average percentage of flowering intensity (Ibrahim and Awang 1992).

5.3.3. Seed collection, seed parameters and germination

Samples of 20 ripe pods were randomly collected from two ramets of each 2x clone and from one or two ramets of all *4x* lines except for BV10L575, which did not produce seed (Table 5.1). Each pod was stored in a separate plastic bag, dried at room temperature, and seeds were extracted from the bag after they had dehisced.

The total number of normal (fully developed and filled) and abnormal (underdeveloped or empty, unfilled, or with wrinkled appearance) seed, as categorised by Nghiem et al (2016), was recorded for each pod. Normal seeds from each pod were also weighed to estimate mean seed weight. These were then scarified and all seeds from each pod germinated in separate petri dishes. The number of germinated seeds was recorded to estimate the germination percentage. Germinated seeds were transplanted to pots containing a mixture of soil, sand and compost mix at nursery condition, then watered daily. Seedlings from each

ramet were grown together in a single tray, and trays were randomised in the nursery in Hanoi, Vietnam, under light shade. Seedling survival was determined after 8 weeks.

Table 5.1. Total number of ramets of diploid (*2x*) and tetraploid (*4x*) acacia hybrid of each genotype used in this study

Genotype/line	Ploidy	Number of ramets assessed in flowering survey	Number of ramets that produced seed	Number of ramets where seed was used
BV33	*2x*	54	6	2
724	*4x*	16	11	1
695	*4x*	19	9	1
684	*4x*	4	2	1
667	*4x*	8	2	1
621	*4x*	6	2	1
BV16	*2x*	41	7	2
437	*4x*	20	9	2
440	*4x*	24	7	2
438	*4x*	6	1	1
BV10	*2x*	57	2	2
583	*4x*	21	2	1
590	*4x*	16	2	1
562	*4x*	19	2	2
575	*4x*	12	0	0
Total		323	62	20

5.3.4. Ploidy determination and microsatellite analysis of progeny

Ploidy was determined for 1348 progenies (*2x* and *4x* F_2), after three months in the nursery. The samples included 474 from diploid trees and 876 from tetraploid mothers. For ploidy determination, nuclei were isolated and stained using the Cystain® DAPI Precise P Kit (Partec, Munster, Germany) with a change to the buffer in order to improve the clarity of results. The kit extraction buffer was replaced with Woody Plant Buffer (Loureiro et al. 2007) with 3 % PVP-10 as detailed by Beatson *et al.* (2003). We determined the ploidy of pooled sample of three seedlings in each flow. Leaf samples from three individual seedlings derived from the same mother and the standard (*Pisum sativum* cv "Torstag") were co-chopped with a double-sided razor blade over ice in a pre-cooled petri dish containing 200 µl WPB2. An additional 200 µl WPB2 was added before filtering through a 20 µm Celltrics® filter (Partec). The suspension was incubated for approximately 2 min with 1.0 ml staining buffer, containing 4',6-diamidino-2-phenylindole (DAPI) prior to analysis. Ploidy of each pooled sample was determined on a Sysmex Partec CyFlow Ploidy Analyser (Goerlitz, Germany) with DNA-DAPI script parameters: L-L 0.30, speed 0.4µl/second and gain ≈ 625 - 635. If a *3x* peak occurred in a bulked sample the three individual DNA samples were to be re-run separately to identify the *3x* individual. This proved to be a quick method to screen a large number of seedlings because the aim was simple, to identify triploids, rather than differentiate aneuploids from true euploids.

After ploidy analysis, 20 progenies per ramet (one seedling selected at random from each pod) were genotyped with SSR markers to estimate outcrossing rates. Phyllodes of the 400 acacia hybrid seedlings derived from the three *2x* clones (BV10, BV16, BV33) (hereafter called *2x* F_2) and 11 induced *4x* lines (hereafter called *4x* F_2) were collected at the nursery, dried with silica gel (or overnight in an oven at 50^{o}C) and stored at room temperature before DNA isolation. DNA was extracted using DNeasy® Plant Mini Kit (Qiagen, Hilden,

Germany), where 20 mg of dried phyllode tissue was crushed to a powder in liquid nitrogen and then the standard Qiagen protocol used for DNA extraction (www.qiagen.com/handbooks). DNA concentration and purity were assessed using gel electrophoresis and comparison with Lambda *Hin*dIII molecular weight standard.

Microsatellite analysis was conducted as described in Le et al. (2016) using 15 of the 16 markers developed to discriminate various hybrid types from pure *A. mangium* and *A. auriculiformis*. The marker AH3_17 was dropped due to poor amplification. PCRs were performed in a GeneAmp PCR system 9700 (Applied Biosystems, USA) with a final volume of 12.5 µl, consisting of 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primer, 0.5U Taq DNA polymerase (Invitrogen, Massachusetts, USA) and 20 ng of genomic DNA. The 15 pairs of SSR primers were multiplexed in four mixes with the annealing temperature (Tm) ranging from 50^{O}C to 60^{O}C depending on marker (Le et al. 2016). The SSR primers were tagged with fluorescent dyes NED, 6-FAM, HEX or ROX on their forward primers and PCR was conducted for all 400 samples. PCR products were separated using an ABI 3730 DNA Analyzer (Applied Biosystems, USA) by the Australian Genome Research Facility (http://www.agrf.org.au). Raw data were analysed using GeneMapper 3.7 (ABI, USA) and Geneious R10 (HTTP:// www.geneious.com) software to score genotypes.

5.3.5. Inheritance of SSR markers in 4x acacia hybrid progenies

Tetraploid F_1 hybrid between species with non-homologous chromosomes are expected to show disomic inheritance where the 2 homologous chromosome sets (e.g., AA and BB) only recombine with their strict homolog (Lloyd and Bomblies 2016). In this case, only 1 gamete (AB) is produced and when this gamete is fertilised with an outcross gamete (e.g., CD) there is only one progeny genotype that can be produced (ABCD). Note that in this case, selfing

can only produce individuals with the AABB genotype (i.e. no homozygous individuals are produced), which cannot be distinguished from the genotype AB using SSR markers. Deviations from disomic inheritance expectations can provide evidence of partial homology between parental genomes resulting in chromosome instability such as tetrasomic inheritance.

In the tetrasomic model four homologues pair at metaphase. In this case, the genotype AABB produces three different diploid gamete genotypes with the allelic composition AA, AB and BB in a ratio of 1:4:1, respectively. When crossing with a different genotype (e.g., CCDD) nine progenies genotypes (AACC, AACD, AADD, ABCC, ABCD, ABDD, BBCC, BBCD, and BBDD) corresponding to nine progenies phenotypes (A_C_, A_CD, A_D_, ABC_, ABCD, ABD_, B_C_, B_CD, and B_D_) will be produced with final ratio of 1:4:1:4:16:4:1:4:1, respectively. If the parents share 1 allele in common, 5 phenotypes will be produced (A_ _ _, A_ _ C, A_B_, AB_C and B_C_) with the ratio of 1:5:5:24:1. When the parents have the same genotype or in cases of selfing, five genotypes (AAAA, AAAB, AABB, ABBB, and BBBB) will be produced and if gene copy number cannot be ascertained (e.g. as with SSRs) these will be detected as three genotypes (A_ _ _, A_ _B and B_ _ _) with the ratio of 1:34:1, respectively. To evaluate the segregation in *4x* F_2, we compared the observed SSR genotypes of 42 outcrossed (see below) progenies with the expected frequencies predicted based on tetrasomic and disomic inheritance models.

5.3.6. Outcrossing rate estimation

The outcrossing rate of each clone was estimated using SSR data and simple exclusion principles (seedlings with non-maternal alleles at two or more loci were declared as outcross) as well as using maximum likelihood method developed for diploids, MLTR (Ritland 1990), and for tetraploids, MLTET (Ritland 2002).

5.3.7. Field trial of F_2 progeny

A progeny trial was established at Ba Vi, Hanoi province, northern Vietnam in 2016 to compare the growth of self and outcross progenies (F_2) of diploid and tetraploid acacia hybrid parent trees. Two hundred and ninety F_2 seedlings genotyped using SSRs survived to the pre-planting stage. These included 214 *4x* F_2 (195 selfed and 19 outcrossed from a total of 10 lines) and 76 *2x* F_2 (33 selfed and 43 outcrossed). Each individual seedlot was represented by between 15 to 30 seedlings laid out in a randomized complete block design with 3 replicates. Each plot contained up to 10 seedlings. Where fewer than 10 genotyped seedlings were available the plots were completed using ungenotyped seedlings from the respective mother tree. All seedlings in the trial were assessed for survival and tree height 12 months after planting but only data from genotyped seedlings were analysed.

5.3.8. Statistical analysis of the Ba vi trial data

One-way ANOVA was used to compare different genotypes and ploidy levels for seed parameters, germination, the survival rate of seedlings at nursery stage and tree height at 12 months after planting into the field trial. These analyses were performed using ANOVA procedures in the R programming language *(*https://www.r-project.org/). To provide adequate sample sizes, data from all lines/clones within each cytotype were pooled in order to test for main cytotype effects.

We used a contingency chi-square test to compare the survival of outcrossed and selfed progenies at each ploidy level. T-test procedures in R were used to compare the height of outcrossed and selfed progenies at each ploidy level. The mean height of outcrossed *4x* and *2x* progenies were compared to the controls (BV10 clone and *A. mangium* seedlings) using post hoc multiple comparisons Tukey's HSD test (α=0.05). The last analysis was conducted

using SPSS (https://www.ibm.com). Inbreeding depression for average height at 12 months after planting was estimated as:

$$\text{ID (\%)} = \frac{(\overline{X}\text{out} - \overline{X}\text{self})}{\bar{X}\text{out}} \text{X } 100$$

where, $\overline{X}$out and $\overline{X}$self are the mean height of seedling originating from outcrossing and selfing, respectively. The significance of inbreeding depression in height between ploidy level was also tested using one-way ANOVA procedures in R.

5.4. Results

5.4.1. Flowering assessments of diploid and tetraploid acacia hybrids

Flowering commenced in late September 2015 and finished in early March 2016. There was variation in the number of ramets that flowered between genotypes and ploidy level. In the diploid (*2x*), only 2 out of 57 BV10 ramets (4%) flowered during the 2015-2106 season, whereas 42% of BV16 and 39% of BV33 ramets flowered (Table 5.1 and Table 5.2). The limited flowering of diploid BV10 was also found in tetraploid (*4x*) BV10, where it had a lower proportion of flowering ramets (26%) than *4x* BV16 (81%) and *4x* BV33 (63%). On average, the proportion of flowering individuals in tetraploid lines (56%) was higher than that in their diploid cytotypes (26%).

There was variation in flowering intensity between genotypes and ploidy levels (Table 5.3). For 2x, the flowering intensity was highest in November (with 26% in BV33 and 28% in BV16) then declined in December to 14% and 20%, respectively. For *4x*, the flowering intensity was highest in January (44% in *4x* BV33 and 49% in *4x* BV16). Overlap of flowering between ploidy levels occurred from early November to late December with a peak of flowering overlap from mid to late December (Table 5.2 and Table 5.3), showing

the potential for open-pollination among clones and between *2x* and *4x* cytotypes in the Bau Bang trial.

Table 5.2. The percentage (%) of ramets of diploid (*2x*) and tetraploid (*4x*) acacia hybrid of each genotype flowering in the Bau Bang trial each month

Genotype	Ploidy	Total number of ramets	Percentage of flowered ramet in each genotype (%)						
			Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.
BV33	*2x*	54	4	18	39	21	6	0	0
	4x	53	0	0	4	25	63	15	1
BV16	*2x*	41	0	12	42	29	7	0	0
	4x	50	0	0	15	44	81	22	3
BV10	*2x*	57	0	0	2	4	2	0	0
	4x	68	0	0	0	9	26	9	1
Mean	*2x*	152	1	13	27	17	5	0	0
	4x	171	0	0	6	26	56	15	2

Table 5.3. Monthly flowering intensity[a] of diploid (*2x*) and tetraploid (*4x*) acacia hybrid genotypes in the Bau Bang field trial

Genotype	Ploidy	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.
BV33	*2x*	2	11	26	14	4	0	0
	4x	0	0	3	21	44	12	1
BV16	*2x*	0	8	28	20	5	0	0
	4x	0	0	7	22	49	11	2
BV10	*2x*	0	0	1	2	1	0	0
	4x	0	0	0	6	17	6	1
Mean	*2x*	1	9	18	11	3	0	0
	4x	0	0	3	16	37	10	1

([a] value expressed as percentage of mean monthly observation for all ramets: <33.3%: little flowering, 33.3-66.6%: moderate flowering and >66.6%: heavy flowering)

5.4.2. Ploidy of open-pollinated progeny from Bau Bang trial

Despite the overlap in flowering time, no triploids were found among the 1348 F_2 progenies that were screened. The ploidy analysis showed that all progenies from *4x* mothers appeared to be *4x*. Similar conservation of ploidy was found in progenies of *2x* mothers, where all progenies were assayed as *2x*.

5.4.3. Seed parameters, germination and survival at nursery stage

There were significant differences in the number of seeds per pod between genotypes as well as significant interactions between genotypes and ploidy (Table 5.4). *2x* BV10 produced fewest seeds per pod whereas *2x* BV33 yielded the most seeds. Similar results were found for the number of normal seeds, with no effect of ploidy but significant differences between genotype and a significant interaction between genotype and ploidy. Genotype, ploidy and the interaction between genotype and ploidy all had significant effects on the number of abnormal seed per pods, but the numbers were low (Table 5.4).

Genotype, ploidy and the interaction between genotype and ploidy all had a significant effect on seed weight. *4x* of each genotype produced larger seeds compared to each of their *2x* progenitors. *4x* BV33 produced the largest seed (19.4 mg), while the smallest seeds were produced by *2x* BV10 (14.2mg). Genotype, ploidy and the interaction between genotype and ploidy had significant effects on seed germination. The percentage of germination was high for all genotype/ploidy combinations (≥80%), however, BV10 had the lowest germination and its *2x* and *4x* lines had very similar germination whereas for the other genotypes there was an advantage of *2x* over *4x*. Ploidy had a significant effect ($P < 0.001$) on seedling survival after 8 weeks in the nursery, while genotype and the interaction between genotype and ploidy had no effect. *4x* progenies had higher survival (47.8 %) than *2x* progenies (34.9%) (Table 5.4).

Table 5.4. Seed parameters, germination and survival of seedlings to 8 weeks from open-pollinated progenies of diploid (*2x*) and tetraploid (*4x*) acacia hybrid genotypes

Genotype	Ploidy	Number of seed per pod	Normal seed/pod[a]	Abnormal seed/pod[b]	Seed weight (mg)[c]	Germination (%)	Survival (%)
BV10	*2x*	3.6	2.9	0.7	14.2	80.0	37.6
	4x	6.7	5.6	1.1	16.5	82.0	42.7
BV16	*2x*	7.0	6.5	0.5	16.3	93.7	32.7
	4x	7.0	6.0	1.0	16.8	85.4	48.7
BV33	*2x*	7.5	7.0	0.5	15.4	94.0	34.4
	4x	6.1	5.2	0.9	19.4	85.0	51.9
Overall	***2x***	***6.0***	***5.5***	***0.6***	***15.2***	***89.2***	***34.9***
	4x	***6.6***	***5.6***	***1.0***	***18.5***	***84.1***	***47.8***
Significance of differences							
Genotype (G)		***	***	***	***	**	ns
Ploidy (P)		ns	ns	***	***	***	***
G X P		***	***	***	***	*	ns

*([a]: number of normal seed per pod, [b]: number of abnormal seed per pod, [c]: calculated for normal seeds only, Asterisks indicate statistical significance *=P<0.05, **= P<0.01, ***= P<0.001)*

5.4.4. Segregation and chromosome instability in tetraploid acacia hybrid

The observed segregation of 15 loci in 42 outcrossed *4x* F_2 is listed in Table 5.5. Among the SSR markers examined, eight were consistent with disomic inheritance. This is because at these eight loci all the outcross F_2 progenies presented the same unique genotype. For seven loci, the observed segregations among F_2 seedlings were in agreement with the expectations for tetrasomic inheritance.

One abnormal genotype was detected at each of three loci (ancp16a&ancp17s, PCT4&ancp8a and Am041), where only one SSR allele was detected; the expectation was for at least two different alleles. These abnormal genotypes were found in three different

seedlings (one for each locus) and the abnormal genotype for PCT4&ancp8a did not have maternal alleles.

Table 5.5. Summary of inheritance pattern for 15 SSR loci in outcross progenies (n = 42) of allotetraploid (*4x*) acacia hybrid

Marker	Mother genotype	Segregation information	Inheritance
Species diagnostic markers			
AH3_6	AABB	3A_ _ _:37A_ B_:2 B_ _ _	Tetrasomic
AH54	AABB	1A_CC:2A_CD:1A_D_:3ABC:22ABCD:5ABD_:2B_C_ :4B_CD:2B_D_	Tetrasomic
AH08	AABB	2A_ _ _:5A_C_:6A_B_:26AB_C:3B_C_ _ _	Tetrasomic
ancp16a&ancp17s	AABB	All A_B_ except for one A_ _ _ *	Disomic, but 1 abnormal (A_ _ _)
ancp54a&ancp55s	AABB	All A_BC	Disomic
Other markers			
AH29	AABB	All A_C_	Disomic
AH69	AABB	All A_B_	Disomic
AH76	AABB	All A_B_	Disomic
AH16	AABB	All ABCD	Disomic
PCT4&ancp8a	AABB	2A_ _ _:6A_C_:5A_B_:25AB_C:3B_C_:1C_ _ _	Tetrasomic, but 1 abnormal (C_ _ _)
ancp29s&ancp30a	AABB	All A_BC	Disomic
ancp69a&ancp70s	AABB	All A_BC	Disomic
Am041	AABB	1A_ _ _:5A_C_:4A_B_:30AB_C:1B_C_:1B_ _ _	Tetrasomic, but 1 abnormal (B_ _ _)
Am387	AABB	2A_ _ _:5A_C_:6A_B_:26AB_C:3B_C_	Tetrasomic
Am465	AABB	1A_CC:2A_CD:1A_D_:3ABC_:25ABCD:3ABD_:2B_C_:3B_CD:2B_D_	Tetrasomic

5.4.5. Outcrossing rates

The outcrossing rate calculated based on the exclusion principle was consistent with those calculated using maximum likelihood (Fig. 5.1), but usually slightly lower (maximum 4% difference between the two methods). Because the varying mode of inheritance of the markers shown above could have affected the outcrossing rates calculated using maximum likelihood, these estimates will not be considered further.

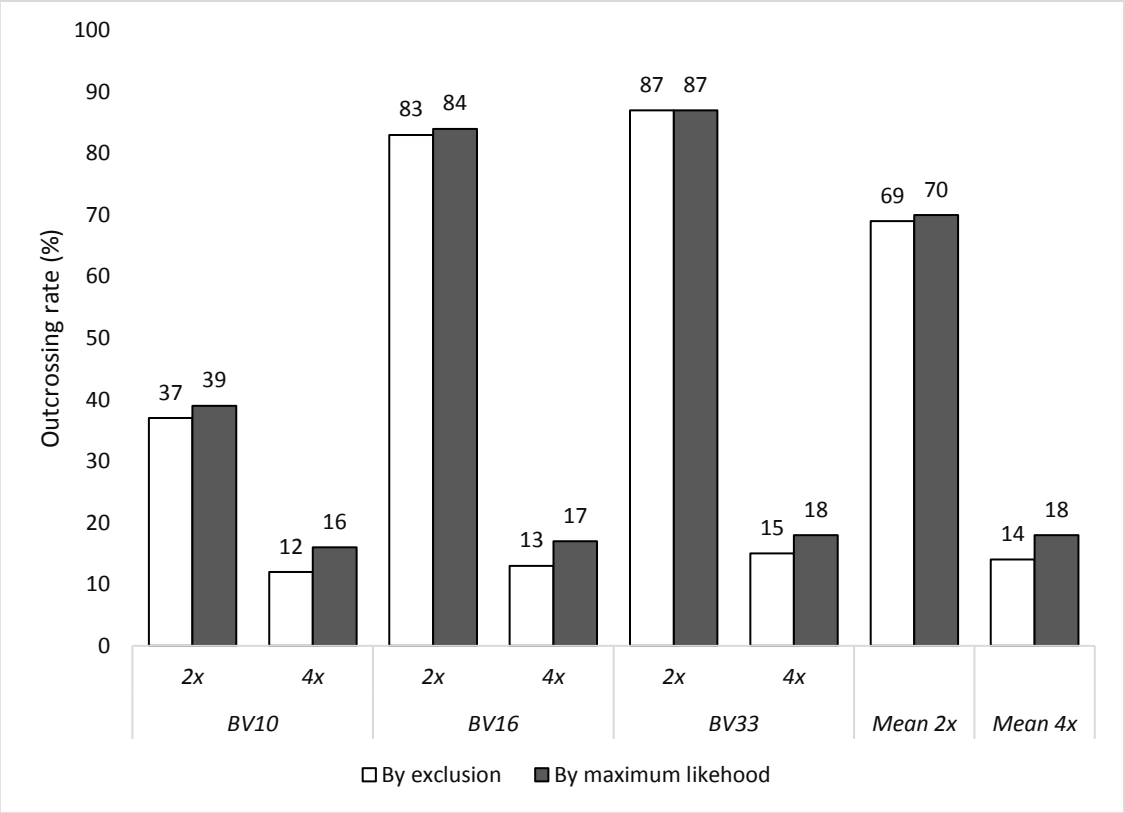


Figure 5.1. Outcrossing rates of diploid (*2x*) and allotetraploid (*4x*) acacia hybrid genotypes

The mean outcrossing rate of *4x* was much lower (14%) than that of *2x* (69%). Among diploids, the outcrossing rate of *2x* BV10 (37%) was lower than that in the other two *2x* genotypes (83% for *2x* BV16 and *2x* 87% for BV33) (Fig 5.1). There was little variation in outcrossing rates among the *4x* genotypes (range of 12 to 15%). No contamination through outcrossing with pollen from outside the trial was found in the progeny of *4x*, whereas in *2x*, 5 (out of 120, approximately 4%) of the seedlings had alleles not found in the three genotypes

within the trial (data not shown). Approximately 80% of outcrosses in both cytotypes were produced by the crossing of BV16 and BV33 (in both directions). The asynchronous flowering of BV10 clone is a likely reason for its low representation in the outcrossed seed.

5.4.6. Survival and height of selfed compared to outcrossed progenies in the field trial

There were significant differences in survival in the field trial between outcrossed and selfed *2x* (Table 5.6). Survival was higher for *2x* outcrossed F_2 (88.5%) than for selfed *2x* F_2 (56.3%). However, there was a no significant difference in the survival of outcrosses (91.4%) and self (86.0%) *4x* (Table 5.6).

Table 5.6. Survival and height growth of outcrosses and self-progenies of *2x* and *4x* acacia hybrid at 12 months in the field trial

Ploidy	Status	Number of planted seedlings	Number survivor	Survival (%)	Height at 12 months (m)	ID[1] (%)	CV[2] (%)
2x	F_2 Outcrosses	61	54	88.5	2.7		25
	F_2 Self	16	9	56.3	1.8	33	28
	Significance (outcrosses vs selfs)			**	**		
4x	F_2 Outcrosses	35	32	91.4	2.9		11
	F_2 Self	178	153	86.0	2.4	17	23
	Significance (outcrosses vs selfs)			*ns*	***		

([1]ID: Inbreeding depression, [2]CV: coefficient variation. Asterisks indicate statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001)

Selfing significantly affected the growth of *2x* and *4x* progenies (Table 5.7). The height of outcrossed F_2 was higher than that of selfed F_2 in both *2x* (2.7 m and 1.8 m, respectively) and *4x* (2.9 m and 2.4 m, respectively). Inbreeding depression of *2x* selfed progenies was significantly ($F = 14.2$, $P = 0.02$) larger (33%) than in *4x* selfed progenies (17%). On average, outcrossed *4x* progenies were more uniform in growth than outcrossed *2x* progenies, coefficients of variation for height being 11% and 25%, respectively.

5.5. Discussion

This study has compared the reproductive biology of allotetraploid acacia hybrid clones and their diploid progenitors allowing for comparison with published information on these traits in autotetraploid of one of the parental species *A. mangium*. The segregation patterns in molecular markers used in this study allow us to draw some novel conclusions about the mixed inheritance pattern in the allotetraploid acacia hybrid. Survival and growth of outcross and self F_2 progenies from both hybrid cytotypes were also investigated in a field trial for the first time.

5.5.1. Flowering, seed production and the breeding system of allotetraploid acacia hybrid in comparison with autotetraploid A. mangium

Nghiem et al. (2011; 2016) described the flowering phenology, seed production and germination characteristics of *2x* and *4x A. mangium*, which is one of the parent species of the acacia hybrid, allowing comparison of these attributes with the acacia hybrid data from our study. Peak flowering in allotetraploid acacia hybrid was approximately one month after peak flowering in their diploid acacia hybrid progenitors. This contrasts with Nghiem et al. (2011) who did not find any significant difference between *A. mangium* cytotypes in

flowering time. This discrepancy is likely caused by differences of genetic composition, because the trial in this study and in Nghiem's study were planted close to one another (5m away). In many other taxa, cytotype differences in flowering time have been reported (Adams and Wendel 2005; Ainouche and Jenczewski 2010; Milan 2008; Ramsey and Schemske 2002).

In our study, there were no significant differences in the number of seeds per pod or number of normal seeds per pod between tetraploid and diploid hybrid cytotypes. In contrast, the *A. mangium* autotetraploid produced half as many seeds per pod compared to diploid cytotypes (Nghiem et al. 2013, Nghiem et al. 2016). In some crop taxa, tetraploid varieties generally produced fewer seeds than diploids (Amdahl et al. 2016; Amdahl et al. 2017; Liatukas and Bukauskaitė 2012; Vleugels et al. 2015). Three possible reasons for low seed yield are proposed: (i) tetraploids produce fewer inflorescences per plant, (Devey et al. 2004) tetraploids have lower pollen viability; and (iii) higher rates of embryo abortion in tetraploids (Vleugels et al. 2015). In our study flowering intensity of tetraploid acacia hybrid was higher than that in its diploid progenitors; we did not study pollen viability or rate of embryo abortion, but it is possible that the autotetraploid *A. mangium* differed from the allotetraploid in these attributes.

There were major differences in outcrossing rate estimate between the two hybrid cytotypes. The diploid clones averaged 69% outcrossing and the tetraploid lines 14%. The diploid estimate is consistent with the 86% reported for acacia hybrid by Ng et al. (2009). There was a large difference in outcrossing rates between clones, BV10 only produced 37% outcrosses, while BV 16 and BV33 had 84% to 87% outcrossing rates, respectively. This can be explained by the phenology data of this clone, where the peak flowering time for this clone is one month later than the other diploid clones in the trial. Therefore, the opportunities for outcrossing were reduced. To analyse the tetraploid data, we pooled lines within

genotypes and the mean values varied only from 12 to 15%. The pattern of variation between cytotypes were similar in autotetraploid *A. mangium* but the difference between cytotypes was even greater. 98% of seeds from the diploid mothers were outcrossed and only 2% of those from 4*x* mothers Griffin et al. (2012). The literature contains many examples of higher selfing (lower outcrossing) in tetraploids compare with equivalent diploids (Rausch et al. 2005; Soltis and Soltis 2000; Stebbins 1971). There are many possible reasons for these differences between cytotypes including: (1) breakdown of gametophytic self-incompatibility (De Nettancourt 2001; Levin 2002) and (2) differences in floral morphology which can affect pollination frequencies (Barrett and Eckert 2012; Miller and Chambers 1993; Stebbins 1971; Tate and Simpson 2004; Webb and Lloyd 1986).

Tetraploid pollen is usually larger than diploid (Baldwin and Husband 2011) and this was found to be the case in the autotetraploid *A. mangium* study of Nghiem et al. (2011). Polyads from *4x* trees were 42 µm in diameter compared with 33 µm in diploid *A. mangium.* However, it was concluded that these differences did not affect the ability of cytotypes to cross-pollinate (Nghiem et al. 2011). In acacia hybrid, the most likely reason for the difference in breeding system of the cytotypes, as measured at seedling stage of the life cycle, is that there is a significant difference in the degree of inbreeding depression expressed in the developing seeds. Selfed diploid seeds may be more likely to abort during development than those from tetraploid (Griffin et al. 2012), increasing the proportion of outcrosses found in the mature open-pollinated seeds of diploids.

It is possible that the outcrossing rate may increase when trees are more mature and produce a higher density of flowers. The small number of genotypes and the design with four ramets of the same genotype per plot may also have influenced the outcrossing rate. Increasing the number of genotypes in a new orchard may increase the flowering overlap and therefore increase the possibility for outcrossing.

Among the 1350 open-pollinated seedlings for which we determined the cytotype we did not identify any *3x* genotypes. In our investigation, it appears that open-pollination is not an efficient method of producing triploids, although previous studies showed that *3x* genotypes can occur at low frequency. When assessing 758 open-pollinated seeds derived from 49 seedlots, collected from a polyploid hybridising orchard at Bau Bang, Vietnam (Griffin et al. 2012), Harbard and Nghiem (2014) found 3 triploid genotypes (frequency of 0.4%). However, based on microsatellite genotyping, these were all derived from *2x* mothers and were likely produced by the fusion of an unreduced and a haploid gamete rather than inter-cytotype outcrossing. It is important to note that our cytotype analyses were conducted on three month old seedlings of normal phenotypes. We cannot discount the possibility that some triploid genotypes were produced but then aborted or failed to germinate or develop normally. It has proved possible to produce viable triploid seedlings by controlled pollination and subsequent in vitro germination techniques (Nghiem et al. 2016).

5.5.2. Mixed inheritance pattern was detected showing the complexity of segregation and recombination in allopolyploid acacia

We analysed the segregation of 15 SSR loci in 42 outcrossed progenies of allotetraploid acacia. Eight SSR loci had segregation consistent with disomic inheritance, while seven loci had segregation consistent with tetrasomic inheritance. This indicates that the allotetraploid cross between *A. mangium* and *A. auriculiformis* does not behave like a 'classic' allopolyploid where chromosomes pair faithfully as bivalents, do not recombine between species sets and display stable disomic inheritance. In fact, it behaves like a segmental polyploid with mixed or intermediate inheritance (De Silva et al. 2005; Stift et al. 2008). This inheritance model was also detected in a polyploid of *Coffea arabica* x *C. canephora* by Lashermes et al. (2000), where nine RFLP markers segregated with tetrasomic

inheritance while two markers followed disomic inheritance. In allotetraploid *Brassica napus*, Grandont et al. (2014) found that only 50% of meiotic cells exclusively showed synaptic bivalents while the rest had one or two synaptic tetravalents per meiosis.

The fact that approximately half the markers showed tetrasomic inheritance may indicate that half the chromosomes form tetravalents at meiosis. However, because the chromosomal positions of the markers are unknown, we do not know how many chromosomes have tetrasomic inheritance, and further research is required to determine how many chromosomes are involved. However, irrespective of the exact number of chromosomes involved, tetrasomic inheritance is likely to result in aneuploidy (Ramsey and Schemske 2002).

A high number of multivalents in polyploids can result in high rates of homologous recombination, which can eliminate the contribution of one parent in a genomic region (Grandont et al. 2013). Therefore, aneuploid (which do not include a whole set of chromosomes) gametes can be produced by tetrasomic inheritance. Aneuploid plants are often less vigorous and fertile than euploids (those with complete chromosome sets) (Comai 2005; Lloyd and Bomblies 2016). Assessments of the genotype data of the outcrossed progenies found three abnormal genotypes, each containing only one allele, which was not consistent with either the disomic or tetrasomic inheritance model. Since no other errors (mislabelling, scoring error) were found with these genotypes, aneuploidy might be considered as the best explanation. The poor growth and survival of the three seedlings (data not shown) are consistent with this explanation. Aneuploids are frequently found in synthetic autotetraploids, for examples, about 30-40% of the progenies of autotetraploid maize are aneuploid (Comai 2005). Because tetraploids generate a large number of aneuploid gametes and progenies, they are also observed to have multisomic rather than disomic inheritance and segregate for parental characteristics in the next generation (Ramsey

and Schemske 2002). Therefore, the fertility of first generation tetraploids is noted to be low. However, fertility has also been observed to improve over several generations in some species (Ramsey and Schemske 2002). This increased fertility is likely the result of increased bivalent formation or disomic inheritance (Ramsey and Schemske 2002). Bivalent pairing of chromosomes in *Nicotiana langsdorfii* x *glauca* increased from approximately 92% at F_2 to almost 100% after 4 generations of fertility selection in F_6 generations (Ramsey and Schemske 2002). This is believed to be due to the fixation of alleles controlling the frequency and the distribution of chiasma (Sybenga 1969). Because we were not able to undertake a cytogenetic study, we do not know how many chromosomes have tetrasomic inheritance and how common aneuploidy is in this acacia polyploid. However, it is likely that after fertility selection these could be improved thereby increasing the chance of finding high performing individuals.

5.5.3. Inbreeding depression in allotetraploids in comparison to diploids

In this study, there was an indication that inbreeding depression was stronger in diploid than in tetraploid acacia hybrids F_2, both in terms of survival and growth after 12 months in a field test. There are good theoretical reasons why this should be the case. Under the dominance hypothesis (Karkkainen et al. 1999), inbreeding depression is believed to occur as a result of the expression of deleterious recessive alleles and as homozygosity for these alleles increases, there is a loss of biological fitness. Under this hypothesis, the lower inbreeding depression in polyploids can be explained by the lower chance of fixation of recessive alleles compared to diploids. However, under the overdominance hypothesis, the doubling of genome size in allotetraploid might cause an increase in heterozygosity (due to fixed differences between species), which may reduce the influence of inbreeding depression in tetraploid progenies (Karkkainen et al. 1999). Under this hypothesis, if all the

chromosomes have bivalent inheritance, and heterozygosity is completely fixed, then inbreeding depression should be absent. In the case of tetraploid acacia hybrids, the presence of a significant but low level of inbreeding depression could be explained by mixed or intermediate inheritance. There is evidence suggesting that diploids are likely to experience more inbreeding depression than tetraploids (Rausch et al. 2005), for example, in *Chamerion angustifolium* (Baldwin and Husband 2013; Husband et al. 2008). In this and other cases, the decrease in inbreeding depression in tetraploids compared to diploids was explained by the over-dominance model (Karkkainen et al. 1999; Ozimec and Husband 2011; Remington and O'Malley 2000).

Despite the lower level of inbreeding depression in tetraploid than in diploid, the total (or population) inbreeding depression estimated as the product of inbreeding depression and the selfing rate, is likely greater in tetraploid due to its high selfing rate. However, since acacia hybrid breeding programs focus on the selection of superior individual genotypes (Harwood et al. 2015), this population level effect is not a major practical problem. Furthermore, tetraploids have several characteristics of high value. These include better fibre length and pulping properties (Griffin et al. 2014; Wang and Cui 2000) as well as superior tree form (less branching, better stem form) (Griffin et al. 2015). Therefore, the proportion of merchantable volume and wood value could be improved in allotetraploids (Griffin et al. 2014). These attributes are yet to be assessed in the allotetraploids. In this study, outcrossed *4x* F_2 showed equivalent growth to outcrossed diploids so prospects are good that superior genotypes can be found and cloned and commercialised directly as suggested by Griffin et al. (2015).

Chapter 6 General discussion and conclusion

In Vietnam, plantations of Australian acacias have expanded rapidly in the past three decades and breeding programs have been established to improve growth, wood properties and disease resistance. Figure 6.1 summarises the genetic improvement strategies being followed in the breeding of tropical acacias in Vietnam and provides a useful framework for considering the application and utility of the SSR markers developed in this thesis.

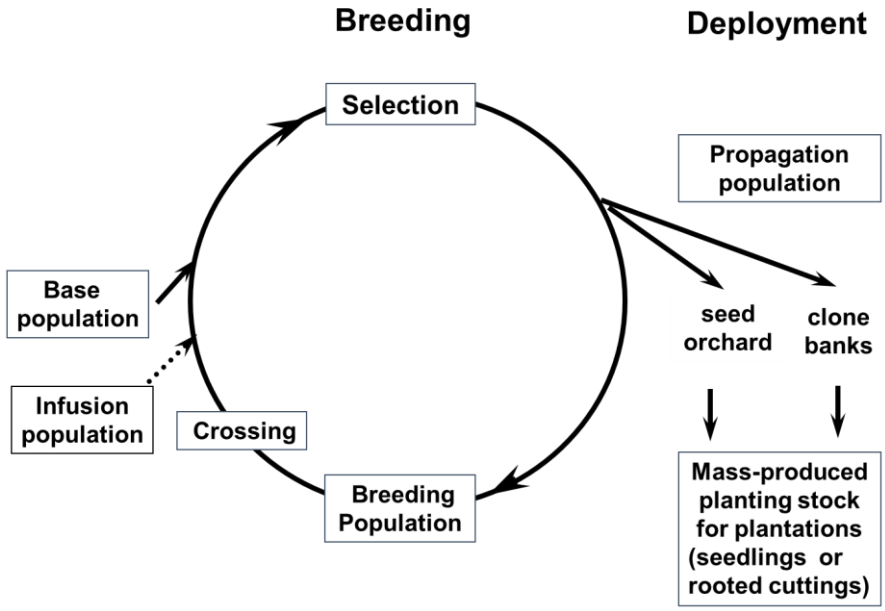


Figure 6.1. Acacia breeding program strategies in Vietnam (Adapted from Kien et al. (2017))

Breeding of tropical acacias in Vietnam commenced in the 1990s. The base populations of both *A. auriculiformis* and *A. mangium* consisted of progeny trials testing over 100 open pollination families from each species. Subsequently, additional introductions (infusion populations) have been used to broaden the breeding populations. The development of superior acacia hybrid clones also commenced in the early 1990s by selecting natural hybrid individuals based on phenotypic superiority (Kha 2001); one area that requires further development is the identification of hybrids among improved parents. Polyploid breeding

of *A. auriculiformis*, *A. mangium* and acacia hybrid is also underway in Vietnam in order to improve adaptability and wood properties as well as produce infertile triploid genotypes (Griffin et al. 2015).

Despite the improvement of controlled pollination methods in tropical *Acacia* (Nghiem et al. 2016), this is still an expensive way of producing diploid interspecific hybrids and triploids; and other methods need investigating, i.e. open-pollination in hybridising orchards (Griffin et al. 2015; Harwood et al. 2015). Other types of polyploids, such as allotetraploids may also offer advantages in facilitating the stable combination of desirable traits from different species. However, little is known about the stability of such polyploids or their reproduction and breeding system. Breeding programs for these taxa now must also pay attention to the issue of cross-contamination between species (Harwood et al. 2015). *Acacia auriculiformis*, *A. mangium* and their selected hybrid clones are the dominant Acacia taxa in Vietnam with no other interbreeding species present (Harwood 2015). In contrast in Brazil where several commercial *Eucalyptus* of subgenus *Symphyomyrtus* are grown together and hybridise freely (Griffin et al. 1988) this simplifies the evaluation of hybridisation using molecular markers. Molecular markers can help to provide solutions to all of these problems, questions or new aspects of acacia breeding and deployment.

This study has identified a set of 16 DNA markers, including ten that are highly polymorphic, in both *A. mangium* and *A. auriculiformis* and six species-diagnostic markers that can distinguish the two species. The markers were used to study current acacia breeding and deployment populations in Vietnam. Application of molecular genetic technologies to the different components of the breeding cycle and deployment is discussed below.

6.1. Characterisation of tropical acacia breeding populations

6.1.1. Genetic diversity and relatedness assessment

Breeding populations can be characterised by quantifying the levels and organisation of genetic variation within and between breeding groups (Raj and Lal 2014). Genetic diversity in breeding populations needs to be monitored in order to avoid the build-up of inbreeding. Using molecular marker data to assess the diversity of breeding population (including base population, elite populations and seed orchards) in comparison with natural populations can enhance the effectiveness of breeding and conservation programs for forest trees, for example in *A. mangium* (Yuskianti and Isoda 2012), *Eucalyptus globulus* (Jones et al. 2006) and *E. grandis* (Okun et al. 2008). In the present study, the genetic diversity of clonal seed orchards (CSOs) of *A. mangium* and *A. auriculiformis* was evaluated, indicated that they were genetically diverse (Chapter 3). However, the genetic diversity of the two pure species CSOs was lower than that in a wide sample of natural provenances. Therefore, there is an opportunity for using the SSR markers as a guide the infusion of new genetic material into Vietnamese acacia breeding populations to prevent relatedness from increasing over generations to the point where inbreeding effects become apparent. In plant breeding, newly imported infusion populations from the natural range and other breeding programs could increase the genetic diversity of breeding populations (Moore et al. 2008). *Acacia mangium* exhibits inbreeding depression (Harwood et al. 2004) and mating among relatives may result in progeny with low performance for economically important traits such as growth, stem form, survival and adaptation. Broadening the genetic base of breeding populations with unrelated infusion materials may also increase the chances of finding genetic tolerance to major diseases, including those that are now a key issue for Vietnam (Nambiar et al. 2015).

Managing the level of heterozygosity or allelic richness may also be of importance to the breeding strategy for advanced generation polyploid acacias. It has been argued that high

heterozygosity may equate with high yield and better adaptation (Alix et al. 2017; Breese et al. 1981; Mason and Batley 2015; Ramsey and Schemske 2002; te Beest et al. 2012). Hence, Griffin et al. (2015) suggested that increasing the number of alleles per locus in *4x* acacia progenies may enhance the potential of finding outstanding polyploid individuals. The set of highly polymorphic SSR markers that were developed here can be used to evaluate the number of alleles per locus in tetraploid genotypes.

Vietnam's breeding populations of *A. auriculiformis* and *A. mangium* were introduced as open-pollinated family seedlots, some of these from the species' natural ranges and others were selected families from breeding programs in Thailand and Australia (Harwood et al. 2015). Pedigree records give some history of provenance and maternal ancestry but gives no information on the paternal side. The use of molecular markers to confirm pedigree information can enhance the effectiveness of selection in breeding and deployment programs (Jones et al. 2006). In this study, the consistency between genetic distances calculated from the SSR markers and known pedigree relatedness shows that the markers used here can provide an accurate and efficient tool for estimating relatedness among acacia individuals of unknown (or partly known) pedigree (Chapter 3).

Dehon et al. (2013) and Rezende et al. (2014) recommended managing genetic diversity and pedigree relatedness in eucalypt hybrid breeding and deployment programs in Brazil to help reduce the risk of disease attack in clonal nurseries and plantations. The same should apply in Vietnam, where only a few hybrid clones are used widely in plantations. In this study (Chapter 3) the new hybrid clones under development in Vietnam displayed higher genetic diversity than the set of current commercial clones, increasing the likelihood that these contain additional genetic variation in economic traits such as disease tolerance. The pedigree information that was obtained from molecular markers in this study could also guide future production and selection of additional unrelated F_1 hybrid genotypes.

6.1.2. Hybrids were found in 'pure' A. auriculiformis and A. mangium populations

The populations of *A. auriculiformis* and *A. mangium* used in breeding programs are not 'pure' because there (1) was hybridisation between them in the wild; and (2) has been contamination events in breeding populations. Genotype assessment performed in this study estimated an approximately 4% of hybridisation between *A. auriculiformis* and *A. mangium* in pure acacia breeding populations in Vietnam (adjacent clonal seed orchards of the two species) (Chapter 2 and Chapter 3). This is consistent with observations in other situations where *A. auriculiformis* and *A. mangium* are grown together in the same habitat (Kha 2001; Sedgley et al. 1992a). Interspecific hybridisation can have positive consequences, for example, increased genetic diversity, fitness, and adaptability (Wang 2001). However, there are also negative effects such as outbreeding depression; which complicate estimations of genetic parameters and breeding values. Using morphological differences for distinguishing parental species and associated hybrids has been shown to be unreliable (Gan and Liang 1992; Le et al. 2017) (Chapter 3). Taxon-specific markers such as those developed in Chapter 2 and used in Chapter 3 together with the pooling DNA technique that were developed in Chapter 4 can now be carried out at low cost (currently about USD30 per individual or pool) therefore, have made an important contribution to the study of interspecific hybridisation and contamination in tropical acacia by reliably distinguishing pure species and hybrid individuals.

6.2. Breeding polyploid acacias

6.2.1. Estimation of mating system parameters and inbreeding depression

In order to assist polyploid acacia breeding in Vietnam, we evaluated the breeding system and reproductive characteristics of induced allotetraploid acacia hybrids. Allotetraploid (*4x*) had a lower level of outcrossing (14%) in comparison with its *2x* progenitors (86% outcrossing), but *4x* selfed progenies were less affected by inbreeding depression than the progenies of the original *2x* cytotypes (Chapter 5). Thus, the total (or population) inbreeding depression estimated as the product of inbreeding depression and the selfing rate is greater in tetraploid due to its high selfing rate. However, because acacia hybrid breeding programs focus on the selection of superior individual genotypes (Harwood et al. 2015), this population level effect is not a major practical problem to breeding. Breeding only needs a low proportion of outstanding individuals for cloning and deployment. The trial results indicated this breeding strategy may be possible as some individuals from both inbred and outcrossed *4x* look promising. In addition, there are a number of studies supporting the efficiency of serially inbreeding plant populations (SIPPs) to purge the deleterious alleles which may cause inbreeding depression (Crnokrak and Barrett 2002). SIPPs can reduce the effects of inbreeding depression in polyploid (Glemin 2003; Lande and Schemske 1985) and Griffin et al. (2015) suggested that this strategy could be used in tropical acacias.

6.2.2. Characterisation of the reproductive mode of polyploid acacia hybrids

Analysis of the mode of inheritance in tetraploid acacias can provide an indication of chromosome pairing behaviours and the recombination potential between the different genomes. The mode of inheritance can be inferred from examinations of the segregation of alleles at a number of individual loci (Lashermes et al. 2000; Stift et al. 2008). The development of the markers used in this study representing various loci with multiple

codominant alleles enabled a preliminary study of the mode of inheritance of allotetraploid acacia hybrid. The allotetraploid acacia hybrid behaves like a segmental polyploid with mixed or intermediate inheritance, as has been found in other species, for example, in tetraploid hybrid coffee (Lashermes et al. 2000), *Brassica napus* (Grandont et al. 2014) and Rorippa (Stift et al. 2008). Importantly, by assessing genotypes of the *4x* offspring, some aneuploid genotypes were detected showing the instability of chromosome behaviour in tetraploid acacia hybrid. Aneuploid plants are often less vigorous and fertile than euploids (those with complete chromosome sets) (Comai 2005; Lloyd and Bomblies 2016). Whether acacia polyploid breeding programs should screen for chromosome stability or whether they should simply screen for growth should be studied. The occurrence of aneuploid seedlings may also have reduced the survival at the nursery of *4x* progenies in allotetraploid acacia hybrid stage in this study.

6.3. Seed orchard design and management, genotype identification

6.3.1. Design and management of pure-species seed orchard and hybridisation orchards

Understanding the pollen dispersal of diploid acacias provides a useful guidance for hybrid seed orchard design and pure seed orchard management (White et al. 2007). Hybrid offspring of *A. auriculiformis* and *A. mangium* were produced at a rate of about 9% in Malaysia through open pollination between adjacent trees of the two species (Wickneswari and Norwati 1992). However, the change in hybrid frequencies with increasing distance from the species boundary was examined for the first time in this study. We used species-diagnostic markers to examine the patterns of hybrid production in adjacent clonal seed orchards of *A. auriculiformis* and *A. mangium* (Chapter 4). As expected, the level of interspecific hybridisation declined significantly with increasing distance from the species boundary. A negative correlation between distance and hybrid frequencies was also detected

in two seed orchards of *A. mangium* (Yuskianti and Isoda 2012), where 80% of cross-pollinations were between trees within 40m of each other. Yuskianti and Isodda (2012) were able to estimate gene flow within single-species seed orchards of *A. mangium* by determining parental identities. Flowering time is one of the main factors affecting the rate of hybrid production. The inferred decline in pollen flow with increasing distance and phenology observation provide useful guidance for the design of hybridising orchards and isolation requirements to prevent contamination of pure-species seed orchards by external pollen sources.

6.3.2. Genotype characterisation, germplasm identification for breeding and clonal forestry

Probably the most important application of molecular markers in tree improvement is the broad problem of germplasm identification. Clone identification in forestry using PCR based markers have been demonstrated in numerous tree species including poplars (Ciftci et al. 2017), willows (Aravanopoulos 2010) and eucalypts (Keil and Griffin 1994; Kirst et al. 2005; Rocha et al. 2002). Microsatellite markers were also used to verify clonal identity and triploid status in *A. dealbata* (Nghiem et al. 2018). The marker set evaluated in the present study showed a very high power for identifying individuals (PI = 8.4×10^{-18} including pure species of *A. mangium*, *A. auriculiformis* and their F_1 hybrid) (Chapter 2). Therefore, it is possible to use these markers to determine the genetic identity of individuals including inter- and intra-specific hybrids, inbred lines and clones, checking mislabelling of ramets/plantlets in clonal propagation, parental examination and validation of controlled crosses in acacia breeding programs. These markers can help to confirm ploidy status – for example, if there are three or more alleles at some loci the individual must be at least a triploid.

6.4. Conclusion

Molecular markers are being applied increasingly to various aspects of tree improvement. This thesis has demonstrated the diverse roles that a set of SSR markers can play in acacia breeding programs. The first application involves assessing the genetic diversity of the acacia breeding populations and potential infusion populations. Ascertaining the origin of and diversity in such populations will help avoid the build-up of inbreeding. Managing genetic diversity is central to the success of breeding programs. A second application is checking the purity status of pure-species breeding populations and confirming the status of interspecific hybrids. A third application is clonal identification. Correct labelling of acacia clones is important especially when the planted genotypes will be used for further analysis (e.g. in linkage mapping and QTL analysis). The use of molecular markers to evaluate the inheritance mode of polyploids reported here is also considered as an alternative method to cytogenetic analysis. In addition, understanding pollen flow in seed orchards by using SSR markers to detect cross-pollination event provides an essential guidance for seed orchard design and management. These show that microsatellite markers will continue to play important roles in tropical acacia breeding programs in the near future.

References

Adams KL, Wendel JF (2005). Polyploidy and genome evolution in plants. Curr Opin Plant Biol 8:135-141

Adams WT, Neale DB, Loopstra CA (1988). Verifying controlled crosses in Conifer tree-improvement programs. Silvae Genet 37:147-152

Adamski DJ, Dudley NS, Morden CW, Borthakur D (2013). Cross-amplification of nonnative *Acacia* species in the Hawaiian Islands using microsatellite markers from *Acacia koa*. Plant Biosyst 147:1088-1091

Adhikari P, Chen LL, Chen X, Sapkota SD, Hwang CF (2014). Interspecific hybrid identification of *Vitis aestivalis*-derived 'Norton'-based populations using microsatellite markers. Scientia Horticulturae 179:363-366

Agarwal M, Shrivastava N, Padh H (2008). Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Reports 27:617-631

Aggarwal RK, Lalremruata A, Allainguillaume J, Consuegra S, Croxford A, Vanhaecke D, Bajay MM, Monteiro M, Pinheiro JB, Barthwal S, Chauhan P, Ginwal HS, Rawat A, Bertolino P, Fernandez-Manjarres JF, Frascaria-Lacoste N, Sannier J, Dalton DL, Kotze A, Den belder E, Elderson J, Esselink GD, Smulders MJM, Diaz-Ferguson E, Douglas MR, Drees M, Meyer EI, Gabler-Schwarz S, De Leaniz CG, Goodisman MAD, Matthews EA, Guo B, Hamilton MB, Power LD, Sheridan CK, Wimp GM, Hayes PK, Hong Y, Li C, Kajita T, Ono J, Takayama K, Tamura M, Kalinowski ST, Vu NV, Keller L, Pearcy M, Koop BF, Messmer AM, Sanderson D, Leese F, Liew WY, Sukganah A, Wickneswari R, Martinelli S, Medlin LK, Moyer GR, Williams AS, John Nelson R, Nguyen TTT, Omoto C, Pavinato VAC, Reusch TBH, Sathe S, Tateishi Y, Witte V, Zucchi MI (2011). Permanent Genetic Resources added to Molecular Ecology Resources Database 1 August 2010 - 30 September 2010. Molecular Ecology Resources 11:219-222

Ahmed MM, Guo H, Huang C, Zhang X, Lin Z (2013). Selection of core SSR markers for fingerprinting upland cotton cultivars and hybrids. Aust J Crop Sci 7:1912-1920

Ainouche ML, Jenczewski E (2010). Focus on polyploidy. New Phytologist 186:1-4

Alcântara BK, Veasey EA (2013). Genetic diversity of teak (*Tectona grandis* L.F.) from different provenances using microsatellite markers. Rev Arvore 37:747-758

Alix K, Gerard PR, Schwarzacher T, Heslop-Harrison JS (2017). Polyploidy and interspecific hybridization: partners for adaptation, speciation and evolution in plants. Annals of Botany 120:183-194

Amdahl H, Aamlid TS, Ergon Å, Kovi MR, Marum P, Alsheikh M, Rognli OA (2016). Seed yield of Norwegian and Swedish tetraploid red clover (*Trifolium pratense* L.) populations. Crop Sci 56:603-612

Amdahl H, Aamlid TS, Marum P, Ergon Å, Alsheikh M, Rognli OA (2017). Seed yield components in single plants of diverse Scandinavian tetraploid red clover populations (*Trifolium pratense* L.). Crop Sci 57:108-117

Anderson EC, Thompson EA (2002). A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160:1217-1229

Aravanopoulos FA (2010). Clonal identification based on quantitative, codominant, and cominant marker data: A comparative analysis of selected willow (*Salix* L.) clones. International Journal of Forestry Research Volume 2010, Article ID 906310

Arntzen JW, Wielstra B, Wallis GP (2014). The modality of nine *Triturus* newt hybrid zones assessed with nuclear, mitochondrial and morphological data. Biol J Linnean Soc 113:604-622

Asif M, Rahman M, Mirza JI, Zafar Y (2009). Parentage confirmation of cotton hybrids using molecular markers. Pakistan Journal of Botany 41:695-701

Assoumane A, Zoubeirou A, Rodier-Goud M, Favreau B, Bezançon G, Verhaegen D (2013). Highlighting the occurrence of tetraploidy in *Acacia senegal* (L.) Willd. and genetic variation patterns in its natural range revealed by DNA microsatellite markers. Tree Genetics & Genomes 9:93-106

Awang K, Taylor D (1993). *Acacia mangium:* Growing and utilization. Winrock International and FAO, Bangkok, Thailand

Baldwin SJ, Husband BC (2011). Genome duplication and the evolution of conspecific pollen precedence. Proc R Soc B Biol Sci 278:2011-2017

Baldwin SJ, Husband BC (2013). The association between polyploidy and clonal reproduction in diploid and tetraploid *Chamerion angustifolium*. Molecular Ecology 22:1806-1819

Barbour R, Potts B, Vaillancourt R (2003). Gene flow between introduced and native *Eucalyptus* species: exotic hybrids are establishing in the wild. Australian Journal of Botany 51:429-439

Barbour RC, Potts BM, Vaillancourt RE (2005). Pollen dispersal from exotic eucalypt plantations. Conservation Genetics 6:253-257

Barbour RC, Potts BM, Vaillancourt RE, Tibbits WN, Wiltshire RJ (2002). Gene flow between introduced and native *Eucalyptus* species. New Forests 23:177-191

Barilani M, Bernard-Laurent A, Mucci N, Tabarroni C, Kark S, Perez Garrido JA, Randi E (2007). Hybridisation with introduced chukars (*Alectoris chukar*) threatens the gene pool integrity of native rock (*A. graeca*) and red-legged (*A. rufa*) partridge populations. Biological Conservation 137:57-69

Barrett SC, Eckert CG (2012). Variation and evolution of mating systems in seed plants. In: Kawano S (ed) Biological Approaches and Evolutionary Trends in Plants. Academic Press, London, UK, p 229.

Barringer BC, Geber MA (2008). Mating system and ploidy influence levels of inbreeding depression in *Clarkia* (Onagraceae). Evolution 62:1040-1051

Barry KM, Mihara R, Davies NW, Mitsunaga T, Mohammed CL (2005). Polyphenols in *Acacia mangium* and *Acacia auriculiformis* heartwood with reference to heart rot susceptibility. J Wood Sci 51:615-621

Beatson RA, Ferguson AR, Weir IE, Graham LT, Ansell KA, Ding H (2003). Flow cytometric identification of sexually derived polyploids in hop (*Humulus lupulus* L.) and their use in hop breeding. Euphytica 134:189-194

Bekkaoui F, Mann B, Schroeder B (2003). Application of DNA markers for the identification and managment of hybrid poplar accessions. Agroforestry Systems 59:53-59

Bellemain E, Taberlet P (2004). Improved noninvasive genotyping method: Application to brown bear (*Ursus arctos*) faeces. Molecular Ecology Notes 4:519-522

Benbouza H, Jacquemin JM, Baudoin JP, Mergeai G (2006). Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechnology, Agronomy, Society and Environment 10:77-81

Blakesley D, Allen A, Pellny TK, Roberts AV (2002). Natural and induced polyploidy in *Acacia dealbata* Link. and *Acacia mangium* Willd. Annals of Botany 90:391-398

Bowen M (1981). *Acacia mangium*: a note on seed collection, handling and storage techniques including some experimental data and information on *A. auriculiformis* and the probable *A. mangium x A. auriculiformis hybrid* in Sabah. Forest Research Centre, Sandakan, Sabah,

Brawner J, Japarudin Y, Lapammu M, Rauf R, Boden D, Wingfield MJ (2015). Evaluating the inheritance of *Ceratocystis acaciivora* symptom expression in a diverse *Acacia mangium* breeding population. Southern Forests 77:83-90

Breese EL, Lewis EJ, Evans GM (1981). Interspecies hybrids and polyploidy. Philosophical Transactions of the Royal Society of London Series B, Biological Sciences 292:487-497

Brockwell J, Searle SD, Jeavons AC, Meigan W (2005). Nitrogen fixation in Acacias: an untapped resource for sustainable plantations, Farm Forestry and Land Reclamation vol 115. ACIAR, Canberra.

Brondani RP, Brondani C, Grattapaglia D (2002). Towards a genus-wide reference linkage map for *Eucalyptus* based exclusively on highly informative microsatellite markers. Molecular genetics and genomics : MGG 267:338-347

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D (1998). Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *E. urophylla*. Theoretical and Applied Genetics 97:816-827

Burczyk J, DiFazio S, Adams W (2004). Gene flow in forest trees: how far do genes really travel? Forest Genetics 11:179

Burdon RD, Aimers-Halliday J (2006). Managing risk in clonal forestry. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 1:1-9

Burgarella C, Lorenzo Z, Jabbour-Zahab R, Lumaret R, Guichoux E, Petit RJ, Soto A, Gil L (2009). Detection of hybrids in nature: application to oaks (*Quercus suber* and *Q. ilex*). Heredity 102:442-452

Butcher P, Glaubitz J, Moran G (1999). Applications for microsatellite markers in the domestication and conservation of forest trees. Forest Genetic Resources 27:34-42

Butcher PA, Decroocq S, Gray Y, Moran GF (2000a). Development, inheritance and cross-species amplification of microsatellite markers from *Acacia mangium*. Theoretical and Applied Genetics 101:1282-1290

Butcher PA, Harwood CE, Quang TH (2004). Studies of mating systems in seed stands suggest possible causes of variable outcrossing rates in natural populations of *Acacia mangium*. Forest Genetics 11:303-309

Butcher PA, McDonald MW, Bell JC (2008). Congruence between environmental parameters, morphology and genetic structure in Australia's most widely distributed eucalypt, *Eucalyptus camaldulensis*. Tree Genetics & Genomes 5:189-210

Butcher PA, Moran GF (2000). Genetic linkage mapping in *Acacia mangium*. 2. Development of an integrated map from two outbred pedigrees using RFLP and microsatellite loci. Theoretical and Applied Genetics 101:594-605

Butcher PA, Moran GF, Bell R (2000b). Genetic linkage mapping in *Acacia mangium* 1. Evaluation of restriction endonucleases, inheritance of RFLP loci and their conservation across species. Theoretical and Applied Genetics 100:576-583

Butcher PA, Moran GF, Perkins HD (1998). RFLP diversity in the nuclear genome of *Acacia mangium*. Heredity 81:205-213

Byrne M (2002). Phylogenetics and the conservation and utilisation of *Acacia* in Western Australia. Conservation Science Western Australia 4:10-18

Byrne M, Marquez-Garcia MI, Uren T, Smith DS, Moran GF (1996). Conservation and genetic diversity of microsatellite loci in the genus *Eucalyptus*. Australian Journal of Botany 44:331-341

Chase M, Kesseli R, Bawa K (1996). Microsatellite markers for population and conservation genetics of tropical trees. American Journal of Botany 83:51-57

Ciftci A, Karatay H, Kücükosmanoğlu F, Karahan A, Kaya Z (2017). Genetic differentiation between clone collections and natural populations of European black poplar (*Populus nigra* L.) in turkey. Tree Genetics & Genomes 13:69

Comai L (2005). The advantages and disadvantages of being polyploid. Nat Rev Gen 6:836-846

Costa V, Monteiro N, Beja-Pereira A, Pérez-González J, Fernández-Llario P, Carranza J, Santos P, Zsolnai A, Anton I, Buzgá J, Varga G (2012). Microsatellite markers for

identification and parentage analysis in the European wild boar (*Sus scrofa*). BMC Research Notes 5

Crnokrak P, Barrett SC (2002). Perspective: purging the genetic load: a review of the experimental evidence. Evolution 56:2347-2358

Cullingham CI, Cooke JEK, Dang S, Coltman DW (2013). A species-diagnostic SNP panel for discriminating lodgepole pine, jack pine, and their interspecific hybrids. Tree Genetics & Genomes 9:1119-1127

Cupertino FB, Leal JB, Corrêa RX, Gaiotto FA (2011). Genetic diversity of *Eucalyptus* hybrids estimated by genomic and EST microsatellite markers. Biologia Plantarum 55:379-382

David RG, Tai TH, Clay HS (2002). Identification of red rice, rice, and hybrid populations using microsatellite markers. Weed Science 50:333-339

De Nettancourt D (2001). Incompatibility and incongruity in wild and cultivated plants. Springer Berlin.

De Silva H, Hall A, Rikkerink E, McNeilage M, Fraser L (2005). Estimation of allele frequencies in polyploids under certain patterns of inheritance. Heredity 95:327-334

de Souza HG, van Sebroech Doria KMAB, Basseto MA, Furtado EL, Rosa DD, Marino CL (2010). Genetic diversity of two *Eucalyptus grandis* nucleus populations. Acta Scientiarum - Agronomy 32:621-625

Dehon G, Resende S, Resende M, Assis T (2013). A roadmap to eucalyptus breeding for clonal forestry. In: Fenning T (ed) Challenges and opportunities for the world's forests in the 21st century. Springer, Dordrecht, Germany, pp 394-424.

Del Castillo RF, Trujillo S (2008). Effect of inbreeding depression on outcrossing rates among populations of a tropical pine. New Phytologist 177:517-524

Depeiges A, Goubely C, Lenoir A, Cocherel S, Picard G, Raynal M, Grellet F, Delseny M (1995). Identification of the most represented repeated motifs in *Arabidopsis thaliana* microsatellite loci. Theoretical and Applied Genetics 91:160-168

Devey ME, Carson SD, Nolan MF, Matheson AC, Te Riini C, Hohepa J (2004). QTL associations for density and diameter in *Pinus radiata* and the potential for marker-aided selection. Theoretical and Applied Genetics 108:516-524

Devey ME, Sewell MM, Uren TL, Neale DB (1999). Comparative mapping in loblolly and radiata pine using RFLP and microsatellite markers. Theoretical and applied genetics 99:656-662

Diallo AM, Nielsen LR, Hansen JK, Ræbild A, Kjær ED (2015). Study of quantitative genetics of gum arabic production complicated by variability in ploidy level of *Acacia senegal* (L.) Willd. Tree Genetics & Genomes 11: 80:13

Dick CW, Etchelecu G, Austerlitz F (2003). Pollen dispersal of tropical trees (*Dinizia excelsa*: Fabaceae) by native insects and African honeybees in pristine and fragmented Amazonian rainforest. Molecular Ecology 12:753-764

Doerksen TK, Herbinger CM (2010). Impact of reconstructed pedigrees on progeny-test breeding values in red spruce. Tree Genetics & Genomes 6:591-600

Doran JC, Turnbull JW (1997). Australian trees and shrubs: species for land rehabilitation and farm planting in the tropics. ACIAR Monograph No. 24. ACIAR, Canberra, Australia.

Dow BD, Ashley MV, Howe HF (1995). Characterization of highly variable (GA/CT) n microsatellites in the bur oak, *Quercus macrocarpa*. Theoretical and Applied Genetics 91:137-141

Echt CS, Deverno LL, Anzidei M, Vendramin GG (1998). Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. Molecular Ecology 7:307-316

Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Molecular Ecology 14:2611-2620

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131:479-491

Faria DA, Mamani EMC, Pappas MR, Pappas GJ, Grattapaglia D (2010). A selected set of EST-derived microsatellites, polymorphic and transferable across 6 species of *Eucalyptus*. Journal of Heredity 101:512-520

Fitzpatrick BM (2012). Estimating ancestry and heterozygosity of hybrids using molecular markers. BMC Evolutionary Biology 12

Fofana IJ, Ofori D, Poitel M, Verhaegen D (2009). Diversity and genetic structure of teak (*Tectona grandis* L.f) in its natural range using DNA microsatellite markers. New Forests 37:175-195

Freeman JS (2006) The application of molecular markers to *Eucalyptus globulus* tree improvement. PhD Thesis, University of Tasmania

Freeman JS, Potts BM, Downes GM, Pilbeam D, Thavamanikumar S, Vaillancourt RE (2013). Stability of quantitative trait loci for growth and wood properties across multiple pedigrees and environments in *Eucalyptus globulus*. New Phytologist 198:1121-1134

Gan E, Liang SB (1992). Nursery identification of hybrid seedlings in open pollinated seedlots. In: Carron LT, Aken KM (eds) Breeding Technologies for Tropical Acacias, ACIAR Proceedings Series, No. 37. ACIAR, Tawau, Sabah, Malaysia, pp 76-87.

Garkava-Gustavsson L, Sehic J, Nybom H, Kolodinska Brantestam A (2008). Molecular characterisation of indigenous Swedish apple cultivars based on SSR and S-allele analysis. Hereditas 145:99-112

Glemin S (2003). How are deleterious mutations purged? Drift versus nonrandom mating. Evolution 57:2678-2687

Glover KA, Kanda N, Haug T, Pastene LA, Øien N, Seliussen BB, Sørvik AGE, Skaug HJ (2013a). Hybrids between common and Antarctic minke whales are fertile and can back-cross. BMC Genet 14:25

Glover KA, Pertoldi C, Besnier F, Wennevik V, Kent M, Skaala T (2013b). Atlantic salmon populations invaded by farmed escapees: Quantifying genetic introgression with a Bayesian approach and SNPs. BMC Genet 14:74

Goudet J (1995). FSTAT (Version 1.2): A computer program to calculate F-statistics. Journal of Heredity 86:485-486

Grandont L, Cuñado N, Coriton O, Huteau V, Eber F, Chèvre AM, Grelon M, Chelysheva L, Jenczewski E (2014). Homoeologous chromosome sorting and progression of meiotic recombination in *Brassica napus*: Ploidy does matter! The Plant Cell 26:1448-1463

Grandont L, Jenczewski E, Lloyd A (2013). Meiosis and its deviations in polyploid plants. Cytogenetis and Genome Research 140:171-184

Grattapaglia D, Vaillancourt RE, Shepherd M, Thumma BR, Foley W, Kulheim C, Potts BM, Myburg AA (2012). Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. Tree Genetics & Genomes 8:463-508

Griffin A, Burgess I, Wolf L (1988). Patterns of natural and manipulated hybridisation in the Genus *Eucalyptus* L'hérit.‐A review. Australian Journal of Botany 36:41-66

Griffin A, Vuong T, Vaillancourt R, Harbard J, Harwood C, Nghiem C, Thinh H (2012). The breeding systems of diploid and neoautotetraploid clones of *Acacia mangium* Willd. in a synthetic sympatric population in Vietnam. Sexual Plant Reproduction 25:257

Griffin AR, Chi NQ, Harbard JL, Son DH, Harwood CE, Price A, Vuong TD, Koutoulis A, Thinh HH (2015). Breeding polyploid varieties of tropical acacias: progress and prospects. Southern Forests 77:41-50

Griffin AR, Midgley SJ, Bush D, Cunningham PJ, Rinaudo AT (2011). Global uses of Australian acacias - recent trends and future prospects. Diversity & Distributions 17:837-847

Griffin AR, Twayi H, Braunstein R, Downes GM, Son DH, Harwood CE (2014). A comparison of fibre and pulp properties of diploid and tetraploid *Acacia mangium* grown in Vietnam. Appita Journal 67:43-49

Griffin AR, Vuong TD, Harbard JL, Wong CY, Brooker C, Vaillancourt RE (2010). Improving controlled pollination methodology for breeding *Acacia mangium* Willd. New Forests 40:131-142

Gross K, Schiestl FP (2015). Are tetraploids more successful? Floral signals, reproductive success and floral isolation in mixed-ploidy populations of a terrestrial orchid. Annals of Botany 115:263-273

Guillemaud T, Broadhurst L, Legoff I, Henery M, Blin A, Ducatillion C, Ferrando N, Malausa T (2015). Development of 23 polymorphic microsatallite loci in invasive silver wattle, *Acacia dealbata* (Fabaceae). Appl Plant Sci 3:4

Gunn B, McDonald M, Gardiner C (1989a). 1998 Seed collections of tropical acacias in Papua New Guinea and North Queensland. Australian Tree Seed Centre, CSIRO Division of Foretry and Forest Product, Canberra

Gunn BV, McDonald MW, Gardiner C (1989b). 1988 seed collections of tropical acacias in Papua New Guinea and North Queensland. Australian Tree Seed Centre, CSIRO Division of Forestry and Forest Products, Canberra, A.C.T.

Hai PH, Duong LA, Toan NQ, Ha TTT (2015). Genetic variation in growth, stem straightness, pilodyn and dynamic modulus of elasticity in second-generation progeny tests of *Acacia mangium* at three sites in Vietnam. New Forests 46:577-591

Hai PH, Harwood C, Kha LD, Pinyopusarerk K, Thinh HH (2008). Genetic gain from breeding *Acacia auriculiformis* in Vietnam. Journal of Tropical Forest Science 20:313-327

Hancock JM (1999). Microsatellites and other simple sequences: genomic context and mutational mechanisms. In: Goldstein DB, Schlötterer C (eds) Microsatellites: Evolution and Applications. Oxford University Press, Oxford, pp 1-9.

Hansen OK, Changtragoon S, Ponoy B, Kjær ED, Minn Y, Finkeldey R, Nielsen KB, Graudal L (2015). Genetic resources of teak (*Tectona grandis* Linn. f.) - strong genetic structure among natural populations. Tree Genetics & Genomes 11:802

Harbard JL, Nghiem C (2014). Production of triploid Acacia seedlings in a polyploid hybridising orchard in Vietnam and early observations of sterility. Paper presented at the Poster presented at the Acacia 2014 "Sustaining the Future of Acacia Plantation Forestry", International Conference, IUFRO Working Party 2.08.07: Genetics and Silviculture of Acacia, Hue, Vietnam, 18-21 March 2014,

Harwood C (2014). Classical genetics and traditional breeding. In: Henry R, Kole C (eds) Genetics, genomics and breeding of Eucalypts. CRC Press, Boca Raton, Floria, pp 12-33.

Harwood C, Thinh HH, Quang TH, Butcher P, Williams E (2004). The effect of inbreeding on early growth of *Acacia mangium* in Vietnam. Silvae Genet 53:65-68

Harwood CE, Hardiyanto EB, Yong WC (2015). Genetic improvement of tropical acacias: achievements and challenges. Southern Forests 77:11-18

Hasenkamp N, Ziegenhagen B, Mengel C, Liepelt S, Schulze L, Schmitt HP (2011). Towards a DNA marker assisted seed source identification: A pilot study in European beech (*Fagus sylvatica L.*). European Journal of Forest Research 130:513-519

Henry IM, Dilkes BP, Tyagi A, Gao J, Christensen B, Comai L (2014). The BOY NAMED SUE quantitative trait locus confers increased meiotic stability to an adapted natural allopolyploid of *Arabidopsis*. The Plant Cell 26:181-194

Huang F, Widyatmoko AYPBC, Zhang BX, Shiraishi S (2005). A diagnosis of acacia hybrids between *A. mangium* and *A. auriculiformis* using codominant SCAR (Sequence Characterized Amplified Region) markers. Nihon Ringakkai Shi 87:149-152

Huang GH, Liang KN, Zhou ZZ, Ma HM (2016). SSR genotyping - Genetic diversity and fingerprinting of teak (*Tectona grandis*) clones. Journal of Tropical Forest Science 28:48-58

Husband BC, Ozimec B, Martin SL, Pollock L (2008). Mating consequences of polyploid evolution in flowering plants: Current trends and insights from synthetic polyploids. International Journal of Plant Sciences 169:195-206

Husband BC, Schemske D, W. (1996). Evolution of the magnitude and timing of inbreeding depression in plants. Evolution 50:54-70

Ibrahim Z, Awang K (1992). Flowering and fruiting phenology of *Acacia mangium* and *Acacia auriculiformis* in Peninsular Malaysia. In: Carron LT, Aken KM (eds) Breeding Technologies for Tropical Acacias. ACIAR Proceedings Series No. 37, vol 37. ACIAR, Tawau, Sabah, Malaysia, pp 45-48.

Ito S (2002). The infection of heartrot and disease severity on several *Acacia* species in SAFODA plantations.

Ito S, Nanis LH (1997). Survey of heartrot on *Acacia mangium* in Sabah, Malaysia. Jpn Agr Res Q 31:65-71

Jarne P, Lagoda PJ (1996). Microsatellites, from molecules to populations and back. Trends Ecol Evol 11:424-429

Jha S, Dick CW (2010). Native bees mediate long-distance pollen dispersal in a shade coffee landscape mosaic. Proceedings of the National Academy of Sciences 107:13760-13764

Jones RC, Vaillancourt RE, Potts BM, Gore PL (2011). Genetic control of flowering time in *Eucalyptus globulus* ssp. *globulus*. Tree Genetics & Genomes 7:1209-1218

Jones TH, Steane DA, Jones RC, Pilbeam D, Vaillancourt RE, Potts BM (2006). Effects of domestication on genetic diversity in *Eucalyptus globulus*. Forest Ecology and Management 234:78-84

Josue J (1992). Preliminary observations on the flowering phenology and seed production in a seedling hybridising orchard of *Acacia mangium* and *Acacia auriculiformis*. In: Carron LT, Aken KM (eds) Breeding Technologies for Tropical Acacias. ACIAR Proceedings Series, vol 37. pp 45-48.

Karhu A, Dieterich JH, Savolainen O (2000). Rapid expansion of microsatellite sequences in Pines. Molecular biology and evolution 17:259-265

Karkkainen K, Kuittinen H, van Treuren R, Vogl C, Oikarinen S, Savolainen O (1999). Genetic basis of inbreeding depression in *Arabis petraea*. Evolution 53:1354-1365

Kato K, Yamaguchi S, Chigira O, Osaka N (2012). Flowering phenology and germination ability of pollens for *Acacia mangium* and *A. auriculiformis*. Silvae Genet 61:228-236

Kato K, Yamaguchi S, Hanaoka S, Chigira O (2014). Comparative study of reciprocal crossing for establishment of acacia hybrids. Journal of Tropical Forest Science 26:469-483

Kawakami T, Backström N, Burri R, Husby A, Olason P, Rice AM, Ålund M, Qvarnström A, Ellegren H (2014). Estimation of linkage disequilibrium and interspecific gene flow in *Ficedula* flycatchers by a newly developed 50k single-nucleotide polymorphism array. Molecular Ecology Resources 14:1248-1260

Kaya N, Isik K, Adams WT (2006). Mating system and pollen contamination in a *Pinus brutia* seed orchard. New Forests 31:409-416

Keil M, Griffin AR (1994). Use of random amplified polymorphic DNA (RAPD) markers in the discrimination and verification of genotypes in *Eucalyptus*. Theoretical and Applied Genetics 89:442-450

Kha LD (2001). Studies on the use of natural hybrids between *A.mangium* and *A. auriculiformis* in Vietnam. Agriculture Publishing House, Hanoi.

Kha LD, Hai ND, Vinh HQ (1997). Clonal tests and propagation options for natural hybrids between *Acacia mangium* and *A. auriculiformis*. In: Turnbull JW, Crompton HR,

Pinyopusarerk K (eds) Recent Developments in Acacia Planting. vol 82. ACIAR, Hanoi, Vietnam, pp 203-210.

Kha LD, Harwood CE, Kien ND, Baltunis BS, Hai ND, Thinh HH (2012). Growth and wood basic density of acacia hybrid clones at three locations in Vietnam. New Forests 43:13-29

Khosravi R, Kaboli M, Rezaei HR (2013). Detecting hybridization between iranian wild wolf (*Canis Lupus Pallipes*) and free-ranging domestic dog (*Canis Familiaris*) by analysis of microsatellite markers. Zoological Science 30:27-34

Kien ND, Harwood C, Le S (2017). Breeding and deployment for wood production. Paper presented at the IUFRO Acacia Conference 2017 "Towards a sustainable future for Acacia plantations", Yogyakarta, Indonesia, 24 - 28 July

Kiran U, Khan S, Mirza KJ, Ram M, Abdin MZ (2010). SCAR markers: a potential tool for authentication of herbal drugs. Fitoterapia 81:969-976

Kirst M, Cordeiro CM, Rezende GDSP, Grattapaglia D (2005). Power of microsatellite markers for fingerprinting and parentage analysis in *Eucalyptus grandis* breeding populations. Journal of Heredity 96:161-166

Kumar S, Richardson TE (2005). Inferring relatedness and heritability using molecular markers in radiata pine. Molecular Breeding 15:55-64

Lande R, Schemske DW (1985). The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. Evolution 39:24-40

Larcombe MJ, Vaillancourt RE, Jones RC, Potts BM (2014). Assessing a Bayesian approach for detecting exotic hybrids between plantation and native Eucalypts. International Journal of Forestry Research 2014. Article ID 650202:13 pp

Lashermes P, Paczek V, Trouslot P, Combes M, Couturon E, Charrier A (2000). Single-locus inheritance in the allotetraploid *Coffea arabica* L. and interspecific hybrid *C. arabica x C. canephora*. Journal of Heredity 91:81-85

Le S, Harwood CE, Griffin RA, Do SH, Ha TH, Ratnam W, Vaillancourt RE (2017). Using SSR markers for hybrid identification and resource management in Vietnamese Acacia breeding programs. Tree Genetics & Genomes 13:102

Le S, Ratnam W, Harwood CE, Larcombe MJ, Griffin RA, Koutoulis A, Harbard JL, Cyer KS, Yee LW, Ha TH, Vaillancourt RE (2016). A multiplexed set of microsatellite

markers for discriminating *Acacia mangium, A. auriculiformis,* and their hybrid. Tree Genetics & Genomes 12:1-10

Leal-Bertioli S, Shirasawa K, Abernathy B, Moretzsohn M, Chavarro C, Clevenger J, Ozias-Akins P, Jackson S, Bertioli D (2015). Tetrasomic recombination is surprisingly frequent in allotetraploid *Arachis*. Genetics 199:1093-1105

Leal-Bertioli SCM, Moretzsohn MC, Santos SP, Brasileiro ACM, Guimarães PM, Bertioli DJ, Araujo ACG (2017). Phenotypic effects of allotetraploidization of wild *Arachis* and their implications for peanut domestication. American Journal of Botany 104:379-388

Lee SS (2004). Diseases and potential threats to *Acacia mangium* plantations in Malaysia. Una sylva 55:31-35

Levin DA (2002). The role of chromosomal change in plant evolution. Oxford University Press.

Lewis PO, Zaykin D (2001). Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c).

Liatukas Ž, Bukauskaitė J (2012). Differences in yield of diploid and tetraploid red clover in Lithuania. Proceedings of the Latvian Academy of Sciences Section B 66:163-167

Liesebach H, Schneck V, Ewald E (2010). Clonal fingerprinting in the genus *Populus* L. by nuclear microsatellite loci regarding differences between sections, species and hybrids. Tree Genetics & Genomes 6:259-269

Lloyd A, Bomblies K (2016). Meiosis in autopolyploid and allopolyploid *Arabidopsis*. Curr Opin Plant Biol 30:116-122

Loureiro J, Rodriguez E, Doležel J, Santos C (2007). Two new nuclear isolation buffers for plant DNA Flow Cytometry: A test with 37 species. Annals of Botany 100:875-888

Ma YP, Tian XL, Wu ZK, Sun WB, Zhang JL (2014). Evidence for natural hybridization between *Primula beesiana* and *P. bulleyana*, two heterostylous primroses in NW Yunnan, China. Journal of Systematics and Evolution 52:500-507

Maslin BR (2015). Synoptic overview of *Acacia* sensu lato (Leguminosae: Mimosoideae) in East and Southeast Asia. The Gardens' bulletin Singapore 67:231-250

Maslin BR, Miller JT, Seigler DS (2003). Overview of the generic status of *Acacia* (Leguminosae : Mimosoideae). Aust Syst Bot 16:1-18

Maslin BR, Stirton CH (1997). Generic and infrageneric classification in *Acacia* (Leguminosae: Mimosoideae): a list of critical species on which to build a comparative data set. Bulletin of the International Group for the Study of Mimosoideae 20:22-44

Mason AS, Batley J (2015). Creating new interspecific hybrid and polyploid crops. Trends in Biotechnology 33:436-441

McNeill J, Turland NJ (2011). Major changes to the Code of Nomenclature - Melbourne, July 2011. Taxon 60:1495-1497

Mendoza HA, Haynes FL (1974). Genetic basis of heterosis for yield in the autotetraploid potato. Theoretical and Applied Genetics 45:21-25

Michalcová V, Dušinský R, Sabo M, Al Beyroutiová M, Hauptvogel P, Ivaničová Z, Švec M (2014). Taxonomical classification and origin of Kamut® wheat. Plant Systematics and Evolution 300:1749-1757

Midgley SJ, Turnbull JW (2003). Domestication and use of Australian acacias: Case studies of five important species. Aust Syst Bot 16:89-102

Mihara R, Barry KM, Mohammed CL, Mitsunaga T (2005). Comparison of antifungal and antioxidant activities of *Acacia mangium* and *A. auriculiformis* heartwood extracts. J Chem Ecol 31:789-804

Milan PR (2008). Chromosome behaviour and fertility in induced polyploids of grain *Amaranths*. Caryologia 61:199-205

Millar MA, Byrne M (2007). Pollen contamination in *Acacia saligna*: Assessing the risks for sustainable agroforestry. WIT Trans Ecol Environ 106:101-110

Millar MA, Byrne M, Nuberg I, Sedgley M (2008). High outcrossing and random pollen dispersal in a planted stand of *Acacia saligna* subsp. *saligna* revealed by paternity analysis using microsatellites. Tree Genetics & Genomes 4:367-377

Millar MA, Byrne M, Nuberg IK, Sedgley M (2012). High levels of genetic contamination in remnant populations of *Acacia saligna* from a genetically divergent planted stand. Restoration Ecology 20:260-267

Millar MA, Coates DJ, Byrne M (2014). Extensive long-distance pollen dispersal and highly outcrossed mating in historically small and disjunct populations of *Acacia*

woodmaniorum (Fabaceae), a rare banded iron formation endemic. Annals of Botany 114:961-971

Miller JM, Chambers KL (1993). Nomenclatural changes and new taxa in *Claytonia* (Portulacaceae) in western North America. Novon 3:268-273

Miller JS, Venable DL (2000). Polyploidy and the evolution of gender Dimorphism in plants. Science 289:2335-2338

Minn Y, Prinz K, Finkeldey R (2014). Genetic variation of teak (*Tectona grandis* linn. F.) in Myanmar revealed by microsatellites. Tree Genetics & Genomes 10:1435-1449

Moncur MW, Mitchell A, Fripp Y, Kleinschmidt GJ (1995). The role of honey bees (*Apis mellifera*) in eucalypt and acacia seed production areas. The Commonwealth Forestry Review 74:350-354

Moore PH, Delmer D, Ming R (2008). Genomics of tropical crop plants. Springer New York.

Moran GF, Muona O, Bell JC (1989a). *Acacia mangium*: A tropical forest tree of the coastal lowlands with low genetic diversity. Evolution 43:231-235

Moran GF, Muona O, Bell JC (1989b). Breeding systems and genetic diversity in *Acacia auriculiformis* and *A. crassicarpa*. Biotropica 21:250-256

Munoz PR, Resende MFR, Huber DA, Quesada T, Resende MDV, Neale DB, Wegrzyn JL, Kirst M, Peter GF (2014). Genomic relationship matrix for correcting pedigree errors in breeding populations: Impact on genetic parameters and genomic selection accuracy. Crop Sci 54:1115-1123

Murphy DJ (2008). A review of the classification of *Acacia* (Leguminosae, Mimosoideae). Muelleria 26:10-26

Nambiar EKS, Harwood CE (2014). Productivity of acacia and eucalypt plantations in Southeast Asia. 1. Bio-physical determinants of production: opportunities and challenges. International Forestry Review 16:225-248

Nambiar EKS, Harwood CE, Kien ND (2015). Acacia plantations in Vietnam: research and knowledge application to secure a sustainable future. Southern Forests:1-10

Neale D, Devey M, Jermstad K, Ahuja M, Alosi M, Marshall K (1992). Use of DNA markers in forest tree improvement research. New Forests 6:391-407

Neale DB, Kremer A (2011). Forest tree genomics: growing resources and applications. Nat Rev Gen 12:111-122

Nei M (1972). Genetic distance between populations. The American Naturalist 106:283-292

Nevill P, Reed A, Bossinger G, Vaillancourt RE, Larcombe M, Ades PK (2008). Cross-species amplification of *Eucalyptus* microsatellite loci. Molecular Ecology Resources 8:1277-1280

Ng CH, Koh SC, Lee SL, Ng KKS, Mark A, Norwati M, Wickneswari R (2005). Isolation of 15 polymorphic microsatellite loci in Acacia hybrid (*Acacia mangium*× *Acacia auriculiformis*). Molecular Ecology Notes 5:572-575

Ng CH, Lee SL, Ng KKS, Muhammad N, Ratnam W (2009). Mating system and seed variation of *Acacia* hybrid (*A. mangium* × *A. auriculiformis*). Journal of Genetics 88:25-31

Nghiem CQ, Griffin AR, Harwood CE, Harbard JL, Le S, Price AJ, Koutoulis A (2018). Occurrence of polyploidy in populations of *Acacia dealbata* Link in Southeast Tasmania and cytotypic variation in reproductive traits. Australian Journal of Botany (In press)

Nghiem CQ, Harbard JL, Griffin AR, Koutoulis A, Harwood CE, Ha TH (2011). Floral phenology and morphology of colchicine-induced tetraploid *Acacia mangium* compared with diploid *A. mangium* and *A. auriculiformis*: Implications for interploidy pollination. Australian Journal of Botany 59:582-592

Nghiem QC, Ha Huy T, Griffin AR, Harbard JL, Koutoulis A, Harwood CE (2016). Seed development following reciprocal crossing among autotetraploid and diploid *Acacia mangium* and diploid *A. auriculiformis*. Australian Journal of Botany 64:20-31

Nghiem QC, Harbard JL, Griffin AR, Koutoulis A, Harwood CE, Ha TH (2013). Pollen-pistil interactions between autotetraploid and diploid *Acacia mangium* and diploid *A. auriculiformis*. Journal of Tropical Forest Science 25:96-110

Nielsen EE, Bach LA, Kotlicki P (2006). HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. Molecular Ecology Notes 6:971-973

Nurtjahjaningsih I (2016). Parent identification in a multi location trial seed orchard of *Acacia mangium u*sing microsatellite markers. Indonesian Journal of Forestry Research 3:19-26

Nurtjahjaningsih ILG, Saito Y, Tsuda Y, Ide Y (2007). Genetic diversity of parental and offspring populations in a *Pinus merkusii* seedling seed orchard detected by microsatellite markers. Bull Tokyo Univ For 118:1-14

Odong TL, van Heerwaarden J, Jansen J, van Hintum TJL, van Eeuwijk FA (2011). Determination of genetic structure of germplasm collections: are traditional hierarchical clustering methods appropriate for molecular marker data?. Theoretical and Applied Genetics:195

Okun DO, Kenya EU, Muluvi GM, Oballa PO, Odee DW (2008). Analysis of genetic diversity in *Eucalyptus grandis* (Hill ex Maiden) seed sources using inter simple sequence repeats (ISSR) molecular markers. African Journal of Biotechnology 7:2119-2123

Omondi S, Machua J, Gicheru J, Hanaoka S (2015). Isolation and characterization of microsatellite markers for *Acacia tortilis* (Forsk.) Hayne. Conservation Genetics Resources 7:529-531

Omondi SF, Dangasuk OG, Odee DW, Cavers S, Khasa DP (2010). Cross-amplification and characterization of polymorphic microsatellite markers from *Acacia (Senegalia) mellifera* and *Acacia brevispica* to *Acacia senegal* (L.) Willd. Silvae Genet 59:285-288

Omondi SF, Odee DW, Ongamo GO, Kanya JI, Khasa DP (2018). Mating patterns of the gum arabic tree (*Acacia senegal synonym Senegalia senegal*) in two different habitats. New Forests 49:53-65

Ozimec B, Husband BC (2011). Effect of recurrent selfing on inbreeding depression and matting system evolution in an autopolyploid plant. Evolution 65:2038-2049

Pacheco IA, Kagevama PY, Wiendl FM, Berti Filho E (1986). Estudo da dispersão de pólen de *Eucalyptus saligna* Smith por abelhas *Apis mellifera* L. utilizando-se o radiofosforo. IPEF, Piracicaba:47-52

Payn K, Dvorak W, Janse BH, Myburg A (2008). Microsatellite diversity and genetic structure of the commercially important tropical tree species *Eucalyptus urophylla*, endemic to seven islands in eastern Indonesia. Tree Genetics & Genomes 4:519-530

Peakall R, Gilmore S, Keys W, Morgante M, Rafalski A (1998). Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other legume genera: implications for the transferability of SSRs in plants. Molecular biology and evolution 15:1275-1287

Peakall R, Smouse PE (2012). GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28:2537-2539

Pinso C, Nasi R (1992). The potential use of *Acacia mangium*× *Acacia auriculiformis* hybrid in Sabah. In: Carron LT, Aken KM (eds) Breeding Technologies for Tropical Acacias, ACIAR Proceedings Series, No. 37, vol ACIAR Proceedings No. 37. ACIAR, Tawau, Sabah, Malaysia, pp 17-21.

Pinyopusarerk K (1990). *Acacia auriculiformis*: an annotated bibliography. Winrock International Institute of Agricultural Development, Australian Centre for International Agricultural Research, Bangkok, Thailand.

Pinyopusarerk K, Williams ER, Boland DJ (1991). Georaphic-variation in seedling morphology of *Acacia auriculiformis* A. Cunn. ex Benth. Australian Journal of Botany 39:247-260

Plant Protection Department (2015). Dispatch Number 2400/BVTV-QLSVGHR dated 01/12/2015 of Plant Protection Department on reporting on a number of emerging pests and prevention results. Vietnam

Porth I, El-Kassaby YA (2014). Assessment of the genetic diversity in forest tree populations using molecular markers. Diversity 6:283-295

Price AJ, Le S, Harbard JL, Griffin AR, Koutoulis A (2014). Ploidy stability in hedge plants of neo-tetraploid Acacia hybrid clones. Paper presented at the Poster presented at the Acacia 2014 "Sustaining the Future of Acacia Plantation Forestry", International Conference, IUFRO Working Party 2.08.07: Genetics and Silviculture of Acacia, Hue, Vietnam, 18-21 March 2014,

Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. Genetics 155:945-959

Rahman MH, Rajora OP (2002). Microsatellite DNA fingerprinting, differentiation, and genetic relationships of clones, cultivars, and varieties of six poplar species from three sections of the genus *Populus*. Genome 45:1083-1094

Raj AJ, Lal SB (2014). Agroforestry: Theory and practices. Scientific Publishers, India.

Ramsey J, Schemske DW (2002). Neopolyploidy in flowering plants. Annual Review of Ecology and Systematics 33:589-639

Randi E (2008). Detecting hybridization between wild species and their domesticated relatives. Molecular Ecology 17:285-293

Rao HX, Patterson B, Potts B, Vaillancourt R (2008). A microsatellite study on outcrossing rates and contamination in an Eucalyptus globulus breeding arboretum. Journal of Forestry Research 19:136-140

Rausch JH, Morgan MT, Husband B (2005). The effect of self-fertilization, inbreeding depression, and population size on autopolyploid establishment. Evolution 59:1867-1875

Raymond M, Rousset F (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248-249

Remington DL, O'Malley DM (2000). Evaluation of major genetic loci contributing to inbreeding depression for survival and early growth in a selfed family of *Pinus taeda*. Evolution 54:1580-1589

Rezende GDS, de Resende MDV, de Assis TF (2014). Eucalyptus breeding for clonal forestry. In: Fenning T (ed) Challenges and opportunities for the World's forests in the 21st Century. Forestry Sciences, vol 81. Springer, Netherlands, pp 393-424.

Richards AJ (1997). Plant Breeding Systems. Chapman & Hall.

Ritland K (1990). A series of FORTRAN computer programs for estimating plant mating systems. Journal of Heredity 81:236-237

Ritland K (2002). Extensions of models for the estimation of mating systems using n independent loci. Heredity 88:221-228

Roberds JH, Bishir JW (1997). Risk analyses in clonal forestry. Canadian Journal of Forest Research 27:425-432

Rocha RB, Muro Abad JI, Pires IE, De Araújo EF (2002). Fingerprint and genetic diversity analysis of *Eucalyptus* spp. genotypes using RAPD and SSR markers. Forest Sciences 62:24-31

Rousset F (2008). genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Molecular Ecology Resources 8:103-106

Rufelds CV (1988). *Acacia mangium, A. auriculiformis* and hybrid *A. auriculiformis* seedling morphology study. Forest Research Center, Sandakan, Malaysia

Santos L, Sabóia E, Almeida D, Abad J, Missiaggia A, Pereira N, Ahnert D, Gaiotto F, Corrêa R (2011). Evaluation of the genetic diversity of a set of parents of *Eucalyptus spp.* by using microsatellite markers to direct matings. BMC Proceedings 5:1

Schlotterer C (2004). The evolution of molecular markers - just a matter of fashion? Natural Review Genetics 5:63-69

Sedgley M, Harbard J, Smith RMM, Wickneswari R, Griffin AR (1992a). Reproductive biology and interspecific hybridisation of *Acacia mangium* and *Acacia auriculiformis* A. Cunn. ex Benth. (Leguminosae: Mimosoideae). Australian Journal of Botany 40:37-48

Sedgley M, Yong WC, Newman V, Harbard J, Smith RM, Ghan KK, Tajuddin A (1992b). Phenology of *Acacia mangium* and *A. auriculiformis* in Australia and Malaysia. In: Carron LT, Aken KM (eds) Breeding Technologies for Tropical Acacias, ACIAR Proceedings Serries, No. 37. ACIAR, Tawau, Sabah, Malaysia, p 36.

Shukor N, Abd Rashid M, Itam K (1994). Karyotypic comparison of *Acacia mangium* Willd., *A. auriculiformis* A. Cunn. ex Benth and their F_1 and F_2 hybrids. Silvae Genet 43:65-68

Smith DN, Devey ME (1994). Occurence and inheritance of microsatellite in *Pinus radiata*. Genome 37:977-983

Soltis P, Soltis DE (2012). Polyploidy and genome evolution. Berlin, Heidelberg : Springer Berlin Heidelberg.

Soltis PS, Soltis DE (2000). The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences 97:7051-7057

Steane DA, Nicolle D, Sansaloni CP, Petroli CD, Carling J, Kilian A, Myburg AA, Grattapaglia D, Vaillancourt RE (2011). Population genetic analysis and phylogeny

reconstruction in *Eucalyptus* (Myrtaceae) using high-throughput, genome-wide genotyping. Molecular Phylogenetics and Evolution 59:206-224

Steane DA, Vaillancourt RE, Russell J, Powell W, Marshall D, Potts BM (2001). Development and characterisation of microsatellite loci in *Eucalyptus globulus* (Myrtaceae). Silvae Genet 50:89-91

Stebbins GL (1971). Chromosomal evolution in higher plants. Edward Arnold Ltd., London.

Stift M, Berenos C, Kuperus P, van Tienderen PH (2008). Segregation models for disomic, tetrasomic and intermediate inheritance in tetraploids: A general procedure applied to Rorippa (Yellow Cress) microsatellite data. Genetics 179:2113-2123

Suharyanto, Shiraishi S (2011). An identification system using multiplex allele-specific PCR assay in the Japanese black pine (*Pinus thunbergii* Parl.). Breed Sci 61:301-306

Sukganah A, Choong CY, Wickneswari R, Russell J, Neale D (2013). Nucleotide sequence analysis of two lignin genes in *Acacia auriculiformis* × *Acacia mangium* hybrid for enhancement of wood pulp quality. Tree Genetics & Genomes 9:1369-1381

Sumathi M, Yasodha R (2014). Microsatellite resources of eucalyptus: Current status and future perspectives. Botanical Studies 55:1-16

Sybenga J (1969). Allopolyploidization of autopolyploids I. Possibilities and limitations. Euphytica 18:355-371

Tarigan M, Roux J, Van Wyk M, Tjahjono B, Wingfield MJ (2011). A new wilt and die-back disease of *Acacia mangium* associated with *Ceratocystis manginecans* and *C. acaciivora sp*. nov. in Indonesia. South African Journal of Botany 77:292-304

Tate JA, Simpson BB (2004). Breeding system evolution in *Tarasa* (Malvaceae) and selection for reduced pollen grain size in the polyploid species. American Journal of Botany 91:207-213

te Beest M, Le Roux JJ, Richardson DM, Brysting AK, Suda J, Kubesova M, Pysek P (2012). The more the better? The role of polyploidy in facilitating plant invasions. Annals of Botany 109:19-45

Thu PQ, Quynh DN, Dell B (2012). *Ceratocystis* sp. causes crown wilt of *Acacia* spp. planted in some ecological zones of Vietnam. Journal of Plant Protection 5:24-30

Thu PQ, Quynh DN, Fourle A, Barnes I, Wingfield M (2014). *Ceratocystis* wilt - a new and serious threat to Acacia plantations in Vietnam: taxonomy and pathogenicity. Paper

presented at the Sustaining the future of acacia plantation forestry. IUFRO Working Party 2.08.07, Hue, Vietnam,

Tsai LM (1988). Studies on *Acacia mangium* in Kemasul Forest, Malaysia. I. Biomass and Productivity. Journal of Tropical Ecology 4:293-302

Turnbull J, Midgley S, Cossalter C (1997). Tropical acacias planted in Asia: an overview. In: Turnbull JW, Crompton HR, Pinyopusarerk K (eds) Recent developments in Acacia planting. ACIAR Proceedings No. 82. ACIAR, pp 14-28.

Turnbull JW (1986). Acacia. In: Turnbull JW (ed) Multipurpose Australian trees and shrubs. Australian Centre for International Agricultural Research (ACIAR), Canberra.

Vaha JP, Primmer CR (2006). Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. Molecular Ecology 15:63-72

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004). micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535-538

Vanden Broeck A, Storme V, Cottrell JE, Boerjan W, Van Bockstaele E, Quataert P, Van Slycken J (2004). Gene flow between cultivated poplars and native black poplar (*Populus nigra L.*): a case study along the river Meuse on the Dutch–Belgian border. Forest Ecology and Management 197:307-310

Vleugels T, Roldán-Ruiz I, Cnops G (2015). Influence of flower and flowering characteristics on seed yield in diploid and tetraploid red clover. Plant Breed 134:56-61

Waits LP, Luikart G, Taberlet P (2001). Estimating the probability of identity among genotypes in natural populations: Cautions and guidelines. Molecular Ecology 10:249-256

Wang W, Hu M (1996). Interspecies hybridization between *Acacia mangium* and *A. auriculiformis* identified by RAPD markers. Taiwan Journal of Forest Science 11:401-407

Wang X, Cui K (2000). The effects of age and multiploidy on fiber characteristics in *Acacia mangium*. Scientia Silvae Sinicae 36:125-130

Wang XS, Szmidt, A E (2001). Molecular markers in population genetics of forest trees. Scandinavian Journal Forest Research 16:199-220

Webb CJ, Lloyd DG (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. herkogamy. New Zealand Journal of Botany 24:163-178

Wheeler N, Jech K (1992). The use of electrophoretic markers in seed orchard research. New Forests 6:311-238

White TL, Adams WT, Neale DB (2007). Forest genetics. CABI Publishing, Cambridge, MA, USA.

Wickneswari R (1989). Use of isozyme analysis in a proposed *Acacia mangium x Acacia auriculiformis* hybrid seed production orchard. Journal of Tropical Forest Science 2:157-164

Wickneswari R, Norwati M (1992). Pod production and hybrid seed yeld of *Acacia mangium* and *Acacia auriculiformis*. In: Carron LT, Aken KM (eds) Breeding Technologies for Tropical Acacias, ACIAR Proceedings Series, No. 37. ACIAR, Tawau, Sabah, Malaysia, pp 57-62.

Wickneswari R, Norwati M (1993). Genetic diversity of natural populations of *Acacia auriculiformis*. Australian Journal of Botany 41:65-77

Widyatmoko AY, Shiraishi S (2003). Species-specific RAPD markers for identification of *Acacia mangium*, *A. auriculiformis* and their hybrid. Kyushu Journal of Forest Researchs 56:66-68

Wong MML, Cannon CH, Wickneswari R (2012). Development of high-throughput SNP-based genotyping in *Acacia auriculiformis x A. mangium* hybrids using short-read transcriptome data. BMC Genomics 13:726-743

Yahya R, Sugiyama J, Silsia D, Gril J (2010). Some anatomical features of an *Acacia* hybrid, *A. mangium* and *A. auriculiformis* growth in Indonesia with regard to pulp yield and paper strength. Journal of Tropical Forest Science 22:343-351

Yong SYC, Choong CY, Cheong PL, Pang SL, Nor Amalina R, Wickneswari R, Harikrishna JA, Mat-Isa MN, Hedley P, Milne L, Vaillancourt R (2011). Analysis of ESTs generated from inner bark tissue of an *Acacia auriculiformis x Acacia mangium* hybrid. Tree Genetics & Genomes 7:143-152

Yuskianti V, Isoda K (2012). Genetic diversity of *Acacia mangium* seed orchard in Wonogiri Indonesia using microsatellite markers. HAYATI Journal of Biosciences 19:141-144

Yuskianti V, Isoda K (2013). Detection of pollen flow in the seedling seed orchard of *Acacia mangium* using DNA marker. Indonesian Journal of Forestry Research 10:31-41

Yuskianti V, Xiang ZB, Shiraishi S (2011a). Development of sequence characterised amplified region (SCAR) markers in *Acacia auriculiformis*. Journal of Tropical Forest Science 23:299-304

Yuskianti V, Xin HF, Xiang ZB, Shiraishi S (2011b). Diagnosis of interspecific hybrids between *Acacia mangium* and *A. auriculiformis* using single nucleotide polymorphism (SNP) markers. Silvae Genet 60:85-92